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Towards Synthetic Peptide Ligation

A thesis submitted in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

At the Chemistry Department of University College London

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DECLARATION

I hereby declare that the work described in this thesis is the work of the author and has not previously been submitted to this or any other university for any other degree.

Tom Morris

December 2007

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Thanks to my wife, Kirstie, my family, all my non-chemistry friends for all the good times outside the lab. Thanks to Luay Hassan for teaching me Arabic and to people who put on parties in or around London. These provided the basis for many an amusing weekend.

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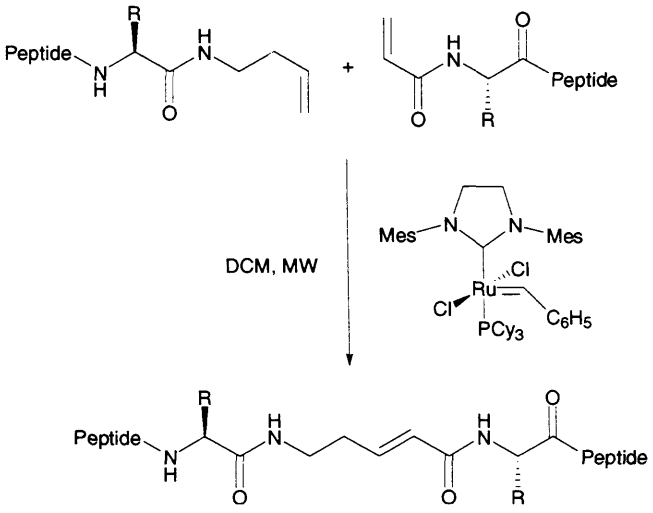
Towards Synthetic Peptide Ligation

ABSTRACT

Presented is a novel protocol for the ligation of protected peptide segments that relies on a selective ruthenium-catalyzed cross metathesis reaction. Amino acid *N*-termini were acryloylated using acryloyl chloride and triethylamine and *C*-termini were coupled to homoallylamine using standard coupling conditions. The conditions used in both reactions are suited to use on protected peptides.



Model studies were performed on a range of single protected amino acids. This demonstrated the applicability of the technique. The synthetic ligation between a protected dipeptide bearing a *C*-terminal homoallyl amide and a protected *N*-acryloyl tripeptide was achieved.

Application to the total chemical synthesis of modified Crambin was then investigated. A site was chosen for ligation and syntheses of the appropriate peptide segments were attempted using solid phases peptide synthesis techniques and an Fmoc protection approach.

CONTENTS

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
Acm	Acetamidomethyl
aq.	Aqueous
Boc	*tert*-Butyloxycarbonyl
Bzl	Benzyl
Cbz, Z	Carboxybenzyl
CM	Cross-metathesis
DCE	Dichloroethane
DCM	Dichloromethane
DIPEA	N,N'-Diisopropylethylamine
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EDC	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl
EDT	Ethane dithiol
EI	Electron impact ionization
ELSD	Evaporative light-scattering detection
equiv.	Equivalents
Fmoc	9-Fluorenylmethyl carbamate
GI	First-generation Grubbs catalyst
GII	Second-generation Grubbs catalyst
GFP	Green fluorescent protein
HATU	2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate
HG	Hoyveda-Grubbs catalyst
HIV	Human immunodeficiency virus
HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography

Im	Imidazole
IR	Infrared
LCMS	Liquid chromatography-mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
Mol%	Mole per cent
mRNA	Messenger RNA
MS	Mass spectrometry
M_w	Molecular weight
MW	Microwave
MWPS	Microwave peptide synthesizer
NCL	Native chemical ligation
NMR	Nuclear magnetic resonance
PG	Protecting group
Phth	Phthalimido
Piv	Pivaloyl
RCM	Ring-closing metathesis
RNA	Ribonucleic acid
ROCM	Ring-opening cross-metathesis
ROMP	Ring-opening metathesis polymerization
RT	Room temperature
SPPS	Solid-phase peptide synthesis
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TIS	Triisopropylsilane
TLC	Thin-layer chromatography
TMS	Trimethyl silane
tRNA	Transfer RNA
Ts	Tosyl

Chapter 1 – INTRODUCTION

1.1 – The Importance of Peptide Synthesis

The new research work presented in this thesis is directed toward the development of new methods for the chemical synthesis of proteins. The importance of having proteins available to us *via* chemical synthesis is twofold. First, the ability to dictate primary structure, which is solely responsible for the protein's higher-order structures, allows, in theory, for total control of protein folding and thus function. This is fundamental to understanding biological systems. Second, the selective modification of a single amino acid allows for the introduction of novel functionality, or site-specific conjugation to a functional structure, such as a nanoparticle[1] or an organic fluorophore.[2] These techniques afford the potential for different proteins to be monitored in isolation or *in vivo*, in turn enhancing our understanding of their function. For example Cole *et al.*, by modification of a single amino acid in tyrosine phosphatase SHP-2, have illuminated the role of this protein in signal transduction;[3] Raines *et al.* have reported the altered folding dynamics and structural stability of RNase A upon insertion of a non-natural amino acid;[4] Muir *et al.*, using a main-chain amide to ester substitution, have explored the factors affecting K^+ conduction properties in potassium channels.[5]

Whilst this ability to manipulate proteins to 'probe nature' is an exciting area, there is a second, less traditional reason for obtaining reliable synthetic protocols to construct and modify proteins. Biological systems are incredibly complex, and protein function is specific often to a point that far surpasses that obtainable in organic chemical systems. It is therefore a goal to harness some aspects of this specificity, which has been perfected in nature over at least 3.5 billion years, and modify it to perform a useful function. For example, the ability of proteins to be integrated into or interfaced with living systems could allow for transport of otherwise toxic molecules by conjugation to the protein at a modified site, and the specificity may result in the ability to deliver this to a particular site within a living organism. Although this is somewhat speculative, it is necessary to highlight the shift in science from observation of the workings of systems in nature to their active manipulation in order to produce highly functional unnatural materials. When

this endeavour incorporates the wealth of functional moieties offered by nature, the potential is great. For example, Davis *et al.* have described the potent inhibition of bacterial aggregation by glycodendrimer-functionalized proteins;[6] DeGrado *et al.*, have synthesized an artificial metalloprotein with potentially tuneable photophysical properties;[7] Poulter *et al.* have developed a technique for site-specific covalent immobilization of proteins on glass slides, applicable to the development of protein chips.[8]

1.2 – The Synthesis of Peptides and Proteins

1.2.1 – Molecular Biology Approaches

Proteins can be effectively produced using recombinant DNA technology, which harnesses the translational hardware of a microorganism such as *E. coli* to produce large quantities of proteins. These are formed by the random cleavage and re-joining of wild-type DNA, amplification and then screening for function of the resultant protein. This technique does not, however, allow for facile access to selective post-translational modifications, such as phosphorylation, which occur in many naturally occurring proteins. Furthermore, in the synthesis of modified proteins with specific functions, site-specific modification of the amino-acid sequence with an unnatural amino-acid is difficult to achieve as it requires transfer RNA that is capable of associating with the amino acid and is compatible with the ribosome during translation. An elegant solution to the problem has been described by Schultz *et al.*[9]

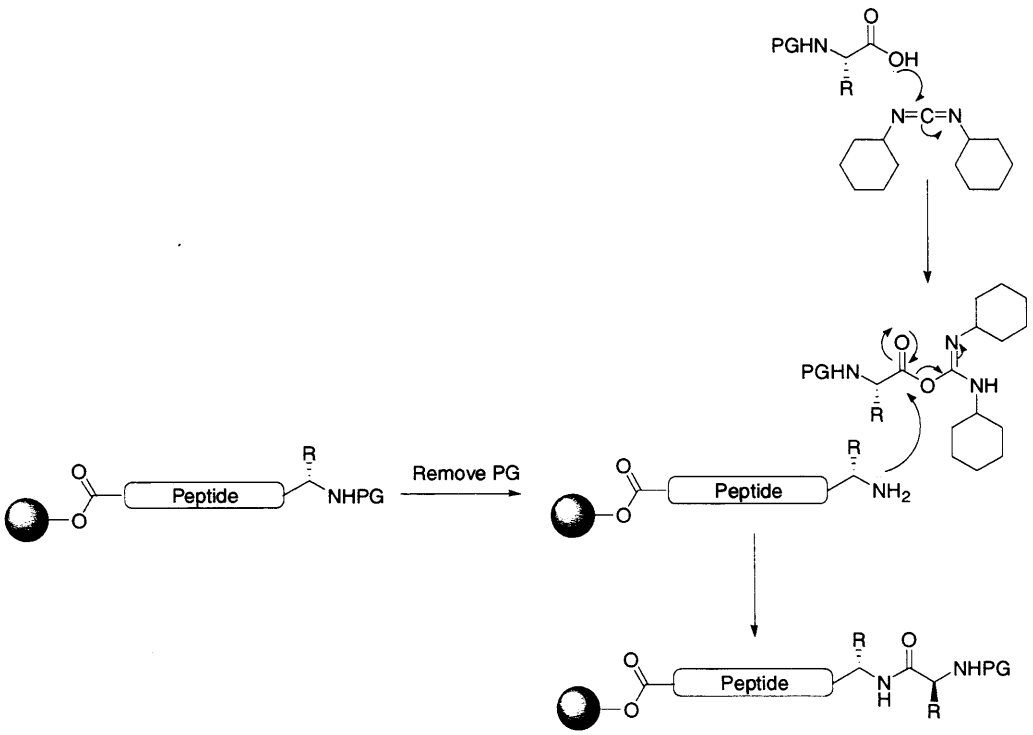
In the Schultz method oligonucleotide-directed mutagenesis is employed to substitute the codon corresponding to the amino acid of interest with a nonsense codon. Then the 3' hydroxyl group of tRNA possessing a complementary anticodon is functionalized with the desired non-natural amino-acid. The functionalized tRNA will therefore associate with the mRNA during *in vitro* translation, thus introducing the non-natural amino acid. This 'cell-free' methodology has been used in a variety of protein modifications. These include preparation of glycopeptides,[10] alteration of luciferase emission wavelengths,[11] increasing fluorescence of green fluorescent protein (GFP)[12] and site specific fluorescent

labelling.[2] Incorporation of non-natural amino-acids has been taken one step further and methods for genetically encoding has been achieved.[13]

1.2.2 – Linear Solid-Phase Synthesis

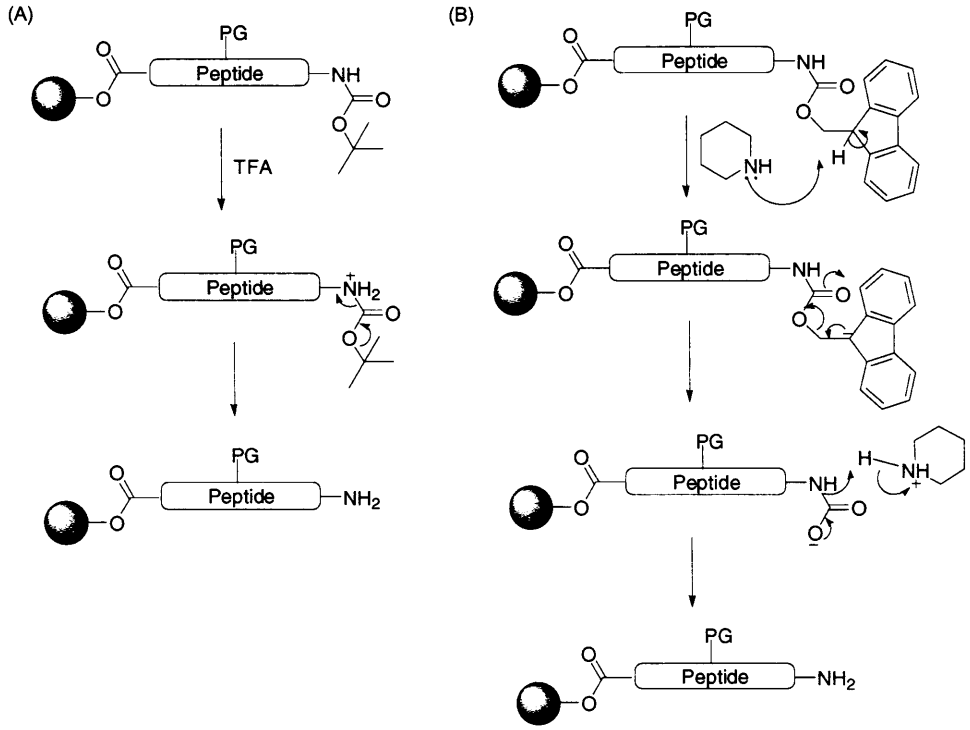
Synthetic chemistry can be used to build peptides sequentially and the field of peptide chemistry has become very reliable, boasting a host of orthogonal protecting groups (PG) and solid-phase techniques.[14] This approach is particularly attractive as it is possible to introduce amino acids in any order, and introduce any number of non-natural amino-acids into the sequence trivially. The ability to access any sequence of amino acids, natural and non-natural is potentially very powerful and could indeed be the foundation of a future 'synthetic biology'.[15]

The basic mechanism of linear peptide synthesis involves nucleophilic attack of the *N*-terminus of the peptide chain at the *C*-terminus of the *N*-protected amino acid to be added (Scheme 1.1). Thus solid phase peptide synthesis (SPPS) usually proceeds with $C \rightarrow N$ directionality. The *N*-terminal protecting group must then be removed, yielding the free amine for subsequent nucleophilic attack. The *C*-terminus, a carboxylic acid, is activated, rendering the carbonyl more susceptible to nucleophilic attack. The *N*-terminal free amine then adds to the carbonyl, producing a tetrahedral intermediate; and the activating group leaves, picking up a proton and forming a urea; and an amide bond is generated. Once the desired number of amino acids has been added, the peptide is cleaved from the resin, usually with removal of side-chain protection simultaneously.



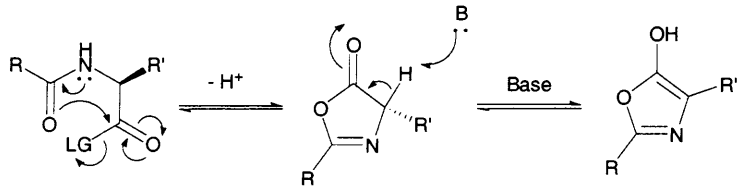
Scheme 1.1 – Solid phase peptide synthesis. PG = protecting group

The two protecting group schemes most widely used are 9-fluorenylmethyl carbamate (Fmoc) and tert-butyloxycarbonyl (Boc), both requiring orthogonal side-chain protection. These are illustrated in Scheme 1.2.



Scheme 1.2 – (A) SPPS using Boc protection. (B) SPPS using Fmoc protection.

The activation of the *C*-terminal carbonyl introduces the possibility of oxazolone rearrangement and subsequent racemization according to Scheme 1.3.



Scheme 1.3 – Mechanism of oxazolone rearrangement

Additives such as copper (II) chloride and 1-hydroxybenzotriazole (HOBt) have been shown to dramatically reduce racemization[16] and HOBt is now commonly used in the synthesis of peptides. Presumably this forms the corresponding ester with the *C*-terminus of the peptide, rendering it less prone to cyclization. Thus if the conversion of the diimide activated species to the HOBt ester is quick enough, racemization is suppressed.

This approach to protein synthesis does, however, have serious limitations. It is restricted to the synthesis of short peptide chains of up to about 50 amino acids, depending on the sequence. This is due to the higher number of deletion peptides, sequences missing amino acids, generated at greater peptide lengths, and the increased difficulty of their removal. Furthermore, at longer lengths the peptide may aggregate into secondary structures, and this could render the *N*-terminus unavailable for reaction.

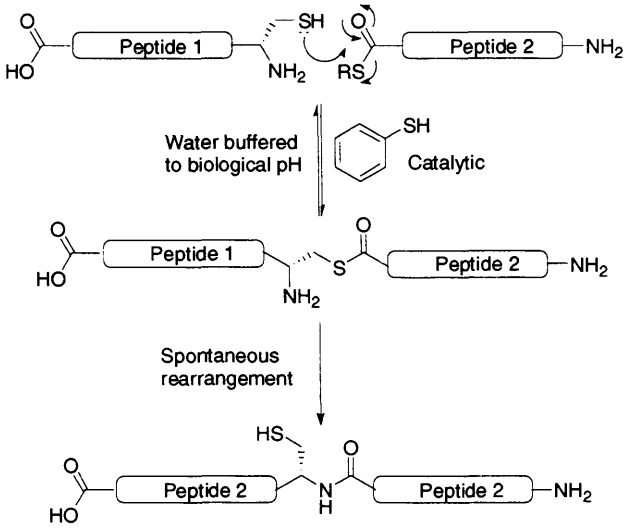
The synthesis of proteins consisting of much longer amino-acid chains therefore requires a convergent approach, which involves first synthesizing shorter peptide segments either by recombinant DNA technology or by synthetic techniques. Such segments are more easily purified, and subsequent joining of these segments can yield peptides with lengths unattainable by the linear synthesis previously described. Using appropriate amino-acid side-chain protection, it is possible to join peptide segments by appropriate synthetic approaches. However side-chain protection often results in poorly soluble peptide segments and associated problems, such as a very low rate of reaction. Convergent synthesis remains very limited.

Given the problems associated with peptide solubility, it is worthwhile considering synthetic techniques which are unaffected by the presence of the functionalities exhibited by the 20 naturally occurring amino acids. Peptide segments could then be joined without the need for protecting groups.

1.3 – Methods of Peptide Ligation

1.3.1 – Native Chemical Ligation – Introduction

Two fully unprotected peptide sequences can be joined in water at or around neutral pH by a process termed Native Chemical Ligation (NCL), first reported by Kent *et al.*[17] in 1994. For it to occur, the *C*-terminus must comprise a thioester and the *N*-terminus a cysteine residue, the sulfhydryl group of which undergoes a reversible transthioesterification reaction with the thioester moiety. In doing this, the free amine is brought into close enough proximity for a spontaneous rearrangement to occur, yielding an amide bond (Scheme 1.4).



Scheme 1.4 – Native chemical ligation

Despite the fact that in-chain unprotected cysteines also possess the sulfhydryl group, the reversible nature of transthioesterification, and the fact the rearrangement is only available to the *N*-terminal cysteine, lead to the reaction being chemoselective.

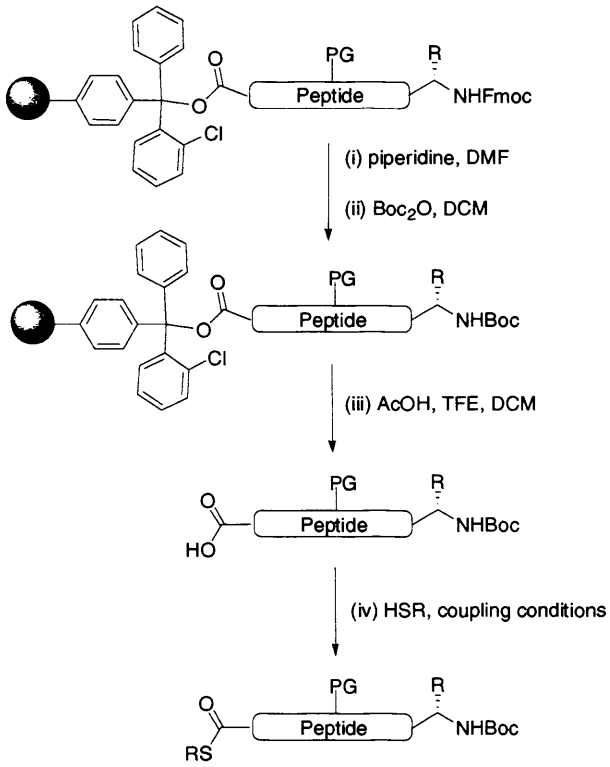
NCL is a reliable technique for synthesizing proteins that are unavailable by linear synthesis, and has been used for the synthesis of many proteins.[18-23] Notably, it has been employed by Kent *et al.* to access the 70 amino-acid potassium channel regulatory subunit KChIP2,[24] a preliminary step towards investigating its role in channel regulation. The same group has used three successive NCL steps in the synthesis of the 130

amino-acid human lysozyme,[25] which allowed for the high-resolution x-ray structure to be determined. They have also reported the total chemical synthesis of the HIV-1 protease dimer,[26] access to which is highly desirable for the development of treatments for AIDS.

NCL has also shown promise in applications beyond the synthesis of native proteins. Bertozzi *et al.*, for example, have used it to lipidate GFP and thus mimic the native glycosylphosphatidylinositol linkage exhibited by membrane-anchored proteins.[27] Additionally, Nagamune *et al.* have described conjugation of biotin and fluorescent proteins to oligonucleotides.[28]

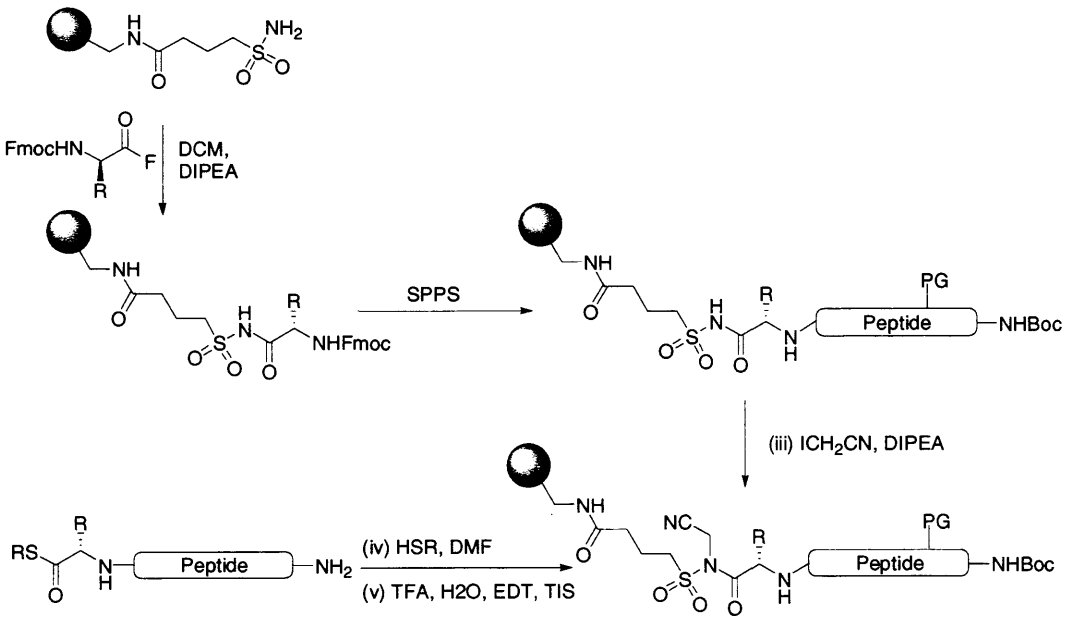
1.3.2 – Native Chemical Ligation – General Procedure

The general procedure is as follows. Peptide segments are synthesized by SPPS as previously described. Peptides requiring *C*-terminal thioester functionalization can be prepared in several ways. These include solution-phase synthesis from fully protected peptide segments, prepared by Fmoc techniques.[20, 21, 29, 30] This approach utilized acid-labile chlorotrityl resins for SPPS of peptides, replacing the *N*-terminal Fmoc for a Boc group, and then cleaved with acetic acid, a milder alternative to TFA which leaves all the protecting groups intact. Thioester generation is then carried out in solution (Scheme 1.5).



Scheme 1.5 – Solution-phase thioester generation

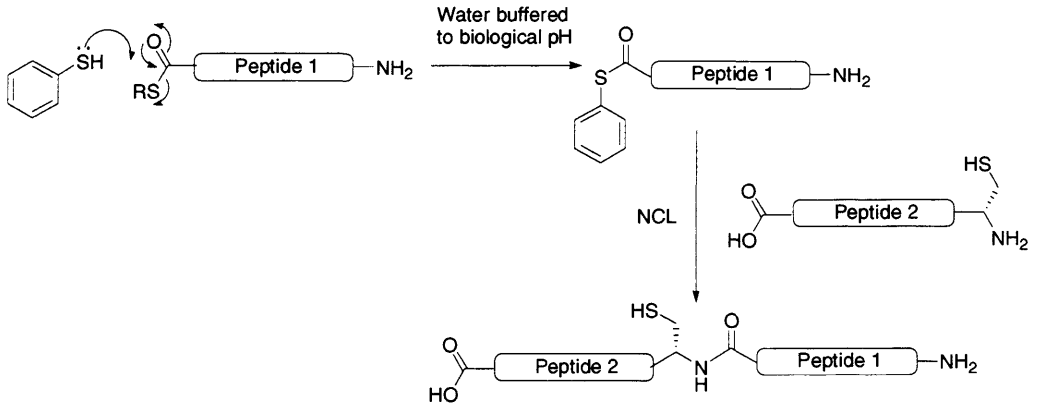
The use of a sulfonamide 'safety-catch' linker has also been reported.[31] Efficient loading of such linkers can be achieved using Fmoc amino-acid fluorides.[32] After SPPS, the group is alkylated and thus converted into a good leaving group. Cleavage with a thiol can therefore be achieved and the resultant thioester deprotected ready for NCL (Scheme 1.6).



Scheme 1.6 – Synthesis of thioesters using a sulfonamide safety-catch linker

It has been shown that the rate of ligation is influenced by the side-chain of the amino acid bearing the thioester moiety;[33] the amino acids resulting in a slow ligation were found to be valine, isoleucine and proline. So this provides yet another limitation, should one of these residues occur next to the cysteine at the chosen ligation site.

NCL commonly requires an additional thiol additive, such as thiophenol, to increase the rate, presumably by providing an additional transthioesterification step yielding the more reactive thioester (Scheme 1.7).[19]

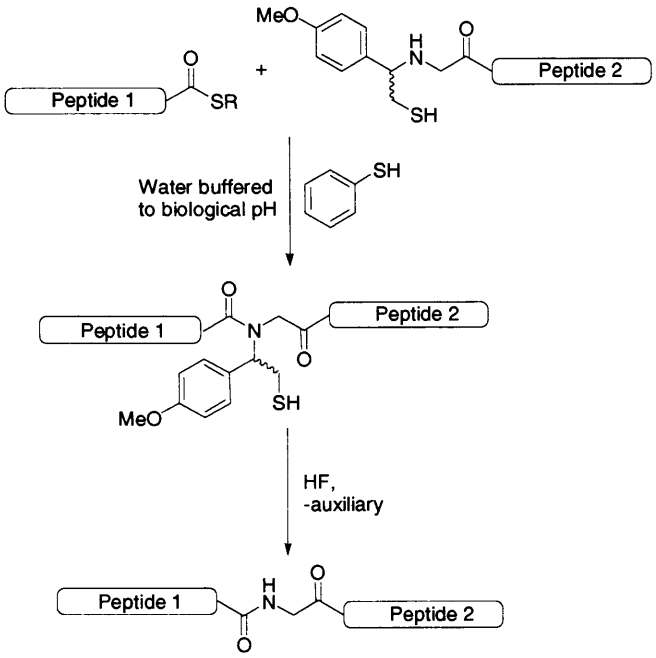


Scheme 1.7 – Mechanism of transthioesterification

Associated with the further activation of the *C*-terminal carbonyl with thiophenol is the danger of racemization *via* the oxazolone rearrangement. The final proteins are almost exclusively characterised by soft ionization mass spectrometry techniques such as MALDI[33] or EI[19, 33, 34] Any racemization occurring during the process will therefore not be detected, and chiral HPLC may have difficulty differentiating between such high M_w diastereoisomers. The function of the protein is also used as a means of characterisation[18] although this is only relevant if a single epimerisation event interferes with biological function.

The issue of racemization has been addressed,[33] by comparison of a small peptide synthesized using NCL in standard aqueous conditions and the same peptide synthesized using standard coupling conditions. Chiral HPLC was employed and the amount of racemization was established to be <2% in the case of the His-Cys ligation. A situation can be easily envisaged in which racemization occurs during ligation at a single site in the polypeptide chain and, due to it being positioned in an area of the protein's tertiary structure not directly involved with the protein's function, has no observable effect. Thus the issue of racemization, whilst being worth addressing, may not actually present a palpable problem in the syntheses of functional peptides using NCL. To achieve academic satisfaction, however, one would wish to be convinced of the preservation of any stereochemistry when using this technique.

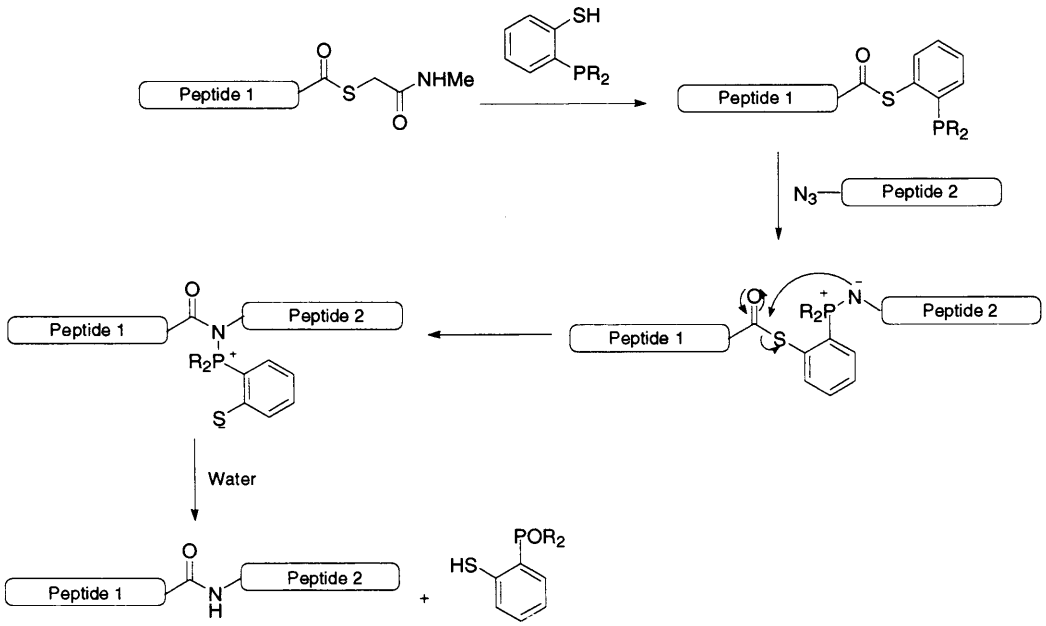
One limitation of NCL is the requirement of a cysteine residue for the reaction to occur. Cysteine occurs with a low frequency relative to other amino acids in both proteins and cyclic peptides. Indeed many peptide sequences do not possess a cysteine at a site, the choice of which would allow division into appropriately sized portions for synthesis and subsequent NCL.[34, 35] One solution relies on a removable auxiliary which mechanistically mimics cysteine and thus undergoes NCL. Removal yields an amide bond at any chosen position.[34, 35] For example utilization of an N^{α}-(1-Phenyl-2mercaptoethyl) derivative (Scheme 1.8), has allowed for generation of cyclic peptides by NCL at non-cysteine containing sites, and the synthesis of cytochrome b562.[34]



Scheme 1.8 – NCL using a removable auxiliary

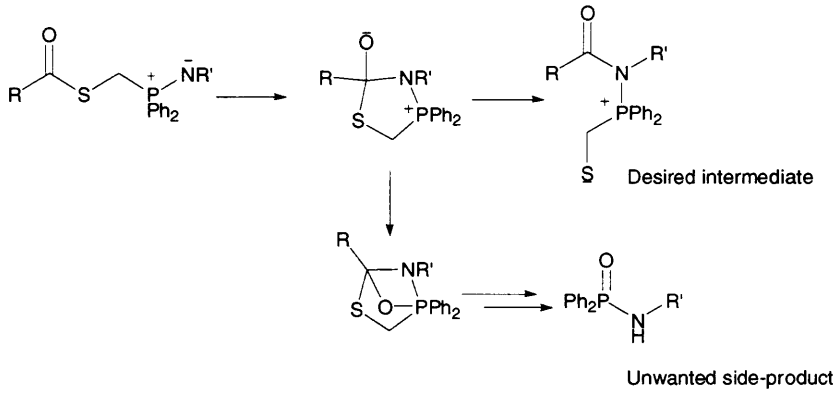
1.3.3 – Other Methods of Peptide Ligation

Due to the inherent drawbacks of NCL, a number of other ligation methods have been developed. The Staudinger ligation (Scheme 1.9) is one such method, and was first reported by Nilsson *et al.*[36] This has no reliance on cysteine residues and, being a modification of the Staudinger reaction, requires one peptide segment to possess an azide moiety. The reaction between this and a thioester in the presence of a phosphinothiol species results in an amide bond and no residual atoms. In essence, the phosphinothiol species acts as a temporary connector.



Scheme 1.9 – Mechanism of the Staudinger ligation

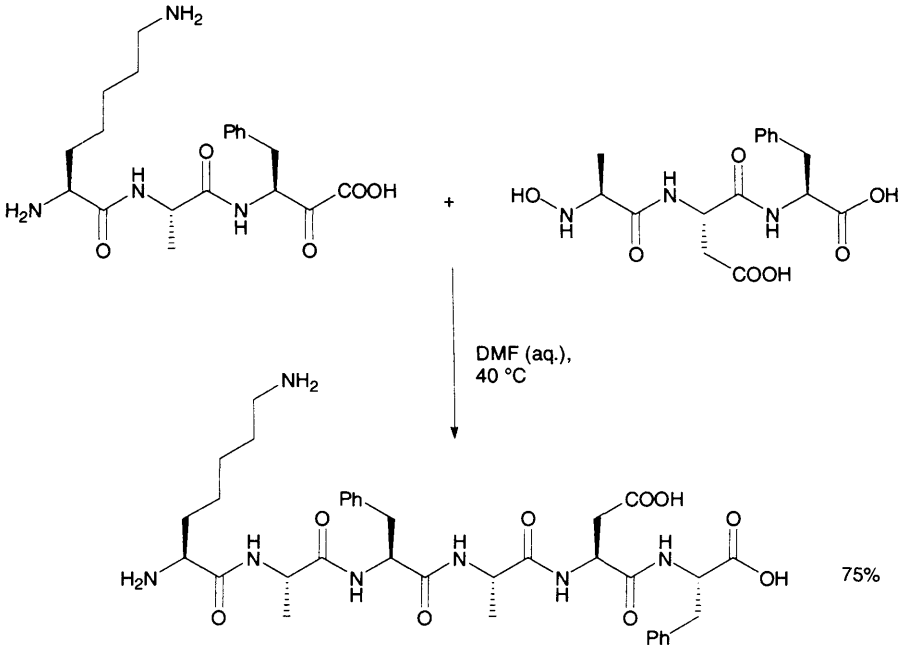
Nilsson *et al.* initially produced a dipeptide using diphenylphosphinobenzenethiol, however the most efficient phosphinothiol species, with ligations proceeding with high (>90%) yields at junctions with a glycine residue at the *N*-terminus, is diphenylphosphinomethanethiol. The yield drops to below 50% in the case of other residues, the by-product being a phosphonamide. A recent study has investigated the effect substitution of the phenyl rings with chlorine and methoxy groups has on the reaction,[37] predicting the electron-donating methoxy groups to increase electron density on the tetrahedral phosphorous intermediate and thus favour the product pathway (Scheme 1.10).



Scheme 1.10 – The two product pathways in the Staudinger ligation

This was indeed found to have the predicted effect, the chloro groups in turn lowering the yield of ligated product. With this improved protocol, an Ala-Ala coupling proceeded with 61% yield in DMF; 99% in toluene. Despite toluene not being a conventional solvent for the manipulation of large peptides, these results are the highest yielding for any non-glycyl Staudinger ligation, and thus are an early indication of what this technique may achieve in the future.

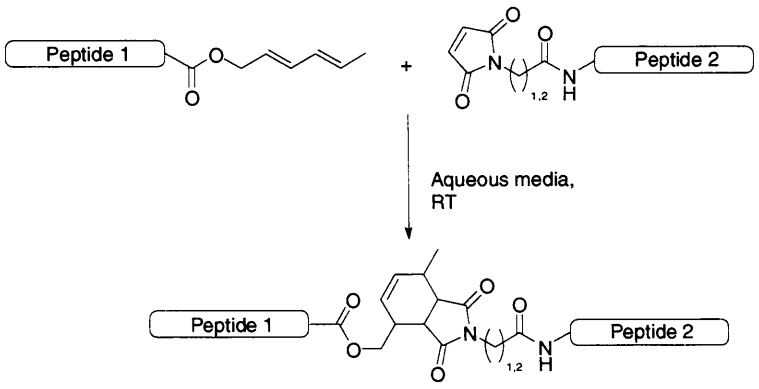
The reaction between α-ketoacids and *N*-alkylhydroxylamines, with the loss of water and carbon dioxide, has been used to ligate small unprotected peptides in DMF, with yields ranging from 58-80% (Scheme 1.11).[38]



Scheme 1.11 – Ligation between an *N*-alkylhydroxylamine and an α-ketoacid

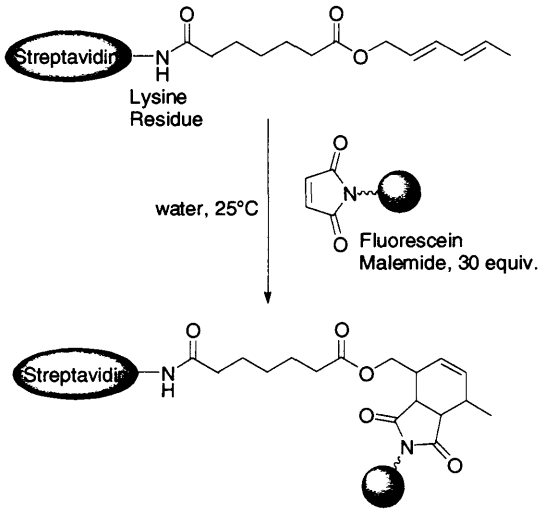
The reaction has also been shown to be applicable to aqueous media, rendering it an attractive option for the semisynthesis of proteins. The mechanism has not yet been determined; however we are informed that this is currently under investigation by Baucom *et al.*, along with a new approach to the preparation of enantiopure ketoacids, which is essential for the general applicability of this methodology.

The Diels-Alder cycloaddition reaction is tolerant of functional groups and occurs under mild conditions, rendering it worthy of the ligation of deprotected peptide fragments. Waldmann *et al.* have ligated a variety of appropriately functionalized short peptides using this cycloaddition,[39] as illustrated in Scheme 1.12.



Scheme 1.12 – Diels Alder ligation

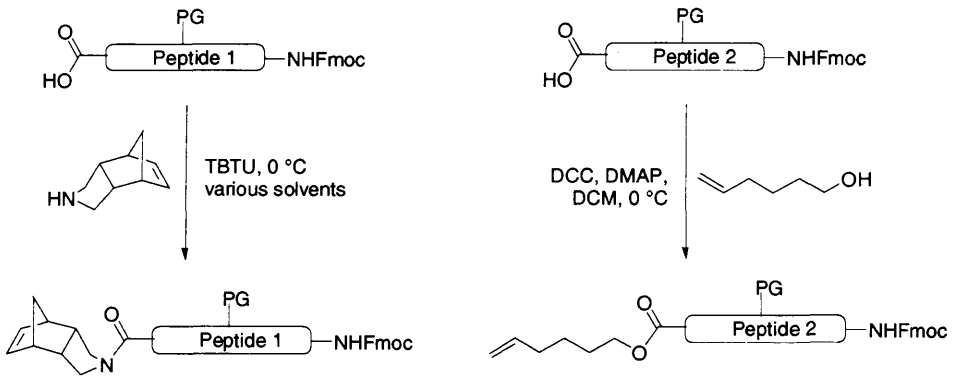
Despite high conversions (>90% except for one case) and good isolated yields (>60% except for one case), this methodology leaves a non-natural linker between peptide segments, which differs significantly from the native peptide structure. For this reason, the technique may be somewhat more worthy as a means to conjugate proteins. This was also achieved in the study of Waldmann *et al*, dansyl and fluorescein-malemide being conjugated to streptavidin (Scheme 1.13) and dansyl-malemide being attached to a Rab protein. The dye-functionalized malemides were attached to streptavidin *via* a lysine residue and to the Rab protein at the *C*-terminus, which had been functionalized appropriately.



Scheme 1.13 – Conjugation *via* Diels Alder ligation

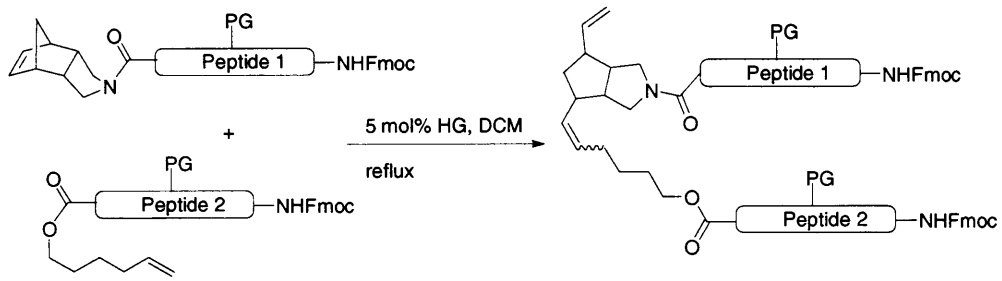
Finally, we turn to a ligation technique that utilizes a ring-opening cross-metathesis (ROCM) reaction.[40] Metathesis is integral to this project, and is discussed in detail in the next section. This study, despite describing a theoretical ligation sensitive to peptide directionality, presents results for ligation between two different amino acids, dipeptides or tripeptides, *C*-terminus to *C*-terminus.

The peptide *C*-termini are first functionalized with olefinic groups of different reactivity and, as shall be discussed in the next section, this leads to selective generation of the required product. This was achieved by coupling the termini to a cyclic, olefinic amine, and hexenol (Scheme 1.14).



Scheme 1.14 – Functionalization of peptide C-termini with olefinic moieties

The two functionalized peptides can now be subjected to metathesis conditions, yielding a longer peptide that contains a non-natural linker and exhibits non-natural directionality (Scheme 1.15). With the exception of one example which yields below 5%, the ROCM reactions yield between 30% and 83% with stereoselectivity of approximately 2:3 *E:Z*.



Scheme 1.15 – Ligation *via* ring opening cross-metathesis

1.4 – Metathesis

1.4.1 - Introduction

Metathesis has had a huge impact on organic chemistry. This is largely because of the technique's generality. Its history, especially the elucidation of the mechanism, is very interesting, however is outside the scope of current discussion. Grubbs, metathesis pioneer and Nobel laureate, has written an excellent historical review on the subject.[41] Metathesis essentially cleaves carbon-carbon double bonds and rejoins them, as shown in Scheme 1.16.

Scheme 1.16 – Olefin metathesis

It relies on a catalytic species, the most frequently used catalysts being ruthenium alkylidenes. Schrock catalysts, usually arylimido complexes of molybdenum, are also used, exhibiting much greater activity at the expense of water and air sensitivity. The ruthenium alkylidene complexes can be weighed out on the bench and so are particularly easy to use. Metathesis is reversible, and thus an equilibrium between starting materials

and products exists in a typical reaction. However, frequently one of the products is ethene which, being volatile, is removed from the reaction mixture thus driving the equilibrium to completion. Metathesis is not restricted to terminal olefins, and in such cases, which are outside the scope of this discussion, it is possible that no volatile by-product is lost. In this case the equilibrium is not driven towards the products in the same way as with terminal olefins.

Examples of the ruthenium alkylidene species responsible for metathesis are the Grubbs first generation catalyst (**GI**), the second-generation catalyst (**GII**)[42] or the Hoyveda-Grubbs catalyst (**HG**),[43] shown in Figure 1.1. The catalytic mechanism first involves the dissociation of one of the ligands. The presence of the cyclic bis-amino carbene species was intended to stabilize the resultant intermediate.[44] Indeed, **GII** and **HG** have greater stability and activity, capable of involving electron-deficient olefins such as acrylic acid derivatives in cross metatheses.[45] **HG** contains a chelating ligand in place of a phosphine, a feature that results in enhanced stability to chromatography and thus greater potential for recovery. Furthermore, the phenyl ring can be substituted in such a way as to fine-tune reactivity.

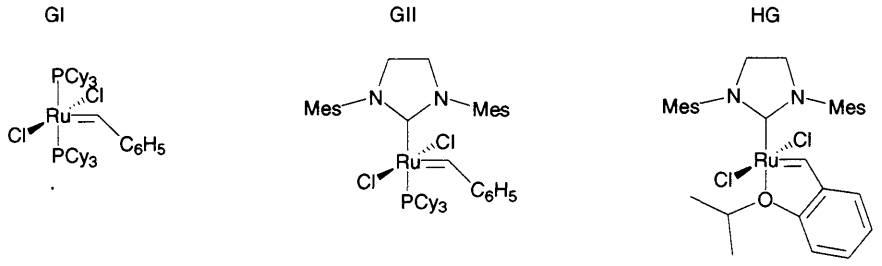
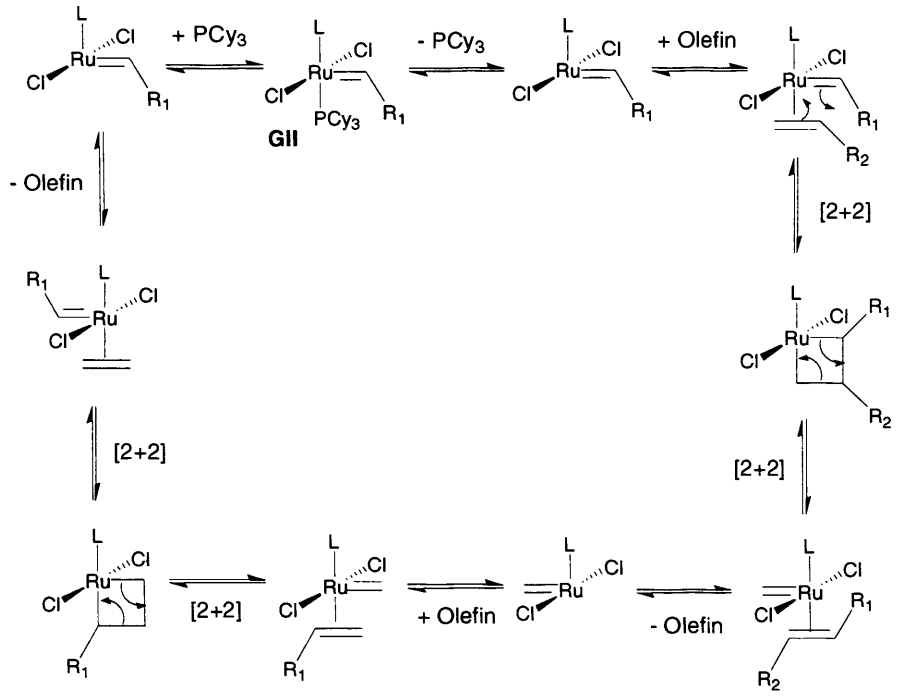


Figure 1.1 – Commercially available ruthenium metathesis catalysts

The mechanism for **GII** (Scheme 1.17) involves the empty coordination site left by the phosphine being replaced by a η^2 coordinated olefin. Next a 2+2 cycloaddition occurs, forming a metallocyclic intermediate with conservation of ruthenium's oxidation state. This process can be reversed either back to the original η^2 coordinated ruthenium complex, or a new olefin is generated, resulting from exchange of alkylidenes. The reversibility of these key steps leads to, essentially, a 'scrambling' of alkylidenes. From

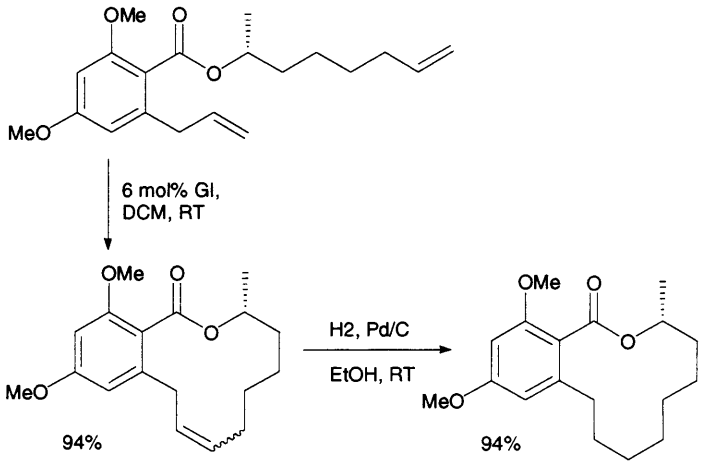
the symmetry of the mechanistic Scheme 1.17 one can see how, in the presence of mixed olefins, the process occurs. Any olefin, which is free to coordinate to the ruthenium, can be split and re-joined.



Scheme 1.17 – The catalytic cycle of metathesis

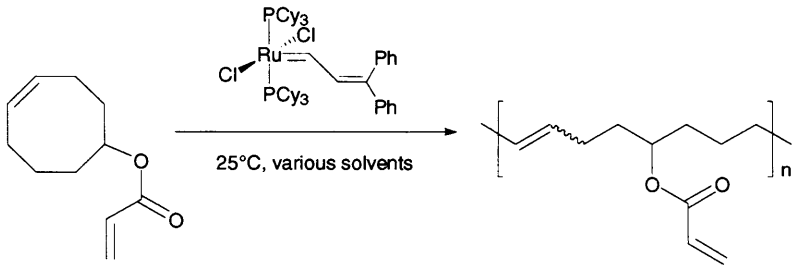
1.4.2 – Ring-Closing Metathesis and Ring-Opening Metathesis Polymerisation

Perhaps the most widely applied class of metathesis is ring-closing metathesis (RCM), *i.e.* metathesis involving the intramolecular cyclisation of two alkenes. In the reaction of two terminal olefins, the release of ethene as a gas in this process is entropically favourable. In essence, two molecules, one of them gaseous, are generated from one. As early as 1996 this was being used as a key step in total syntheses, for the example in the generation of a 12-membered ring in the enantiomerically pure total synthesis of Lasiodiplodin,[46] as shown in Scheme 1.18.



Scheme 1.18 – Employment of RCM in the total synthesis of Lasiodiplodin

If a number of rings are subjected to metathesis conditions, they can open and polymerize in a process termed ring-opening metathesis polymerisation (ROMP). Any ring strain in the precursor will render the process enthalpically favourable, and as an ethene molecule is generated per reactant molecule, the process remains entropically favoured. The process is illustrated with an early example of the polymerization of functionalized cyclo-octenes by Grubbs utilizing a ruthenium alkylidene complex (Scheme 1.19).[47]

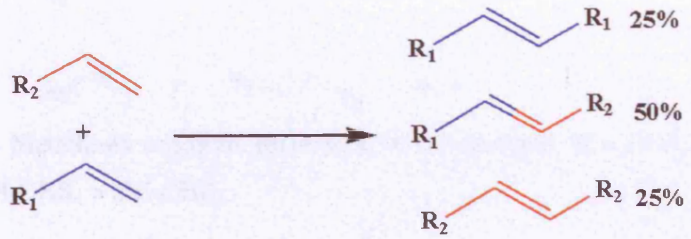


Scheme 1.19 – ROMP of a cyclooctene

More recently, ROMP polymers bearing amino-acid groups have been synthesized from the metathesis of strained cyclobutene-glycine derivatives using the 3rd generation Grubbs catalyst,[48] which shall not be discussed in detail here.

1.4.3 – Cross Metathesis

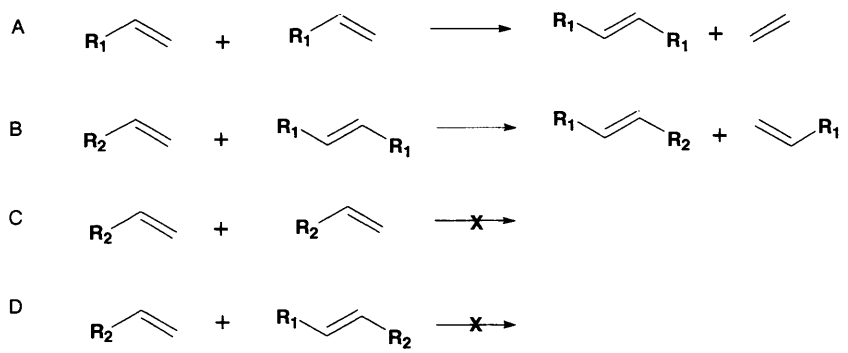
Far less developed than RCM and ROMP is cross-metathesis (CM), *i.e.* metathesis between two olefins contained in different molecules. In the case of the molecules being the same, the reaction is referred to as a self cross-metathesis or homodimerization. In this thesis the terms homodimerization and self cross-metathesis will be used to distinguish these processes from cross-metathesis, *i.e.* the metathesis between two *different* olefins. Scheme 1.20 shows the reaction between two olefins of similar reactivities yielding a statistical mixture of products.



Scheme 1.20 – Cross metathesis reaction performed in the presence of a Grubbs catalyst.

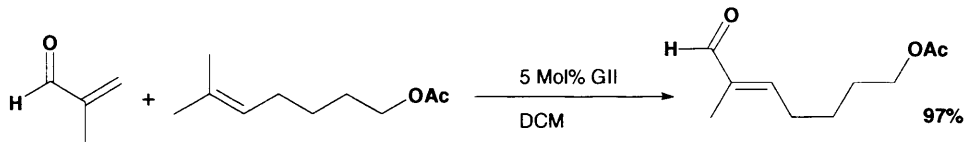
Of course, this would be far from ideal in a synthetic context in which a single product is usually required. Changing relative equivalents of starting materials naturally can provide a method for improving the product distribution. This is inefficient; however reasonable selectivity can be achieved by using olefins of *different* reactivities. It possible to obtain high yields of a single product in the metathesis of appropriate olefin combinations. In a review dedicated to the issue of selectivity in cross-metathesis, Chatterjee *et al.* [49] define the key factor in predicting of the outcome of a cross-metathesis to be the ability of each olefin to homodimerize. Olefins can be divided into those that undergo rapid homodimerization (Type I), those which homodimerize slowly (Type II), those that do not homodimerize (Type III) and those that are not involved in any metathesis events (Type IV). If the two olefins required for cross-metathesis undergo metathesis and are from different groups, selective cross-metathesis can occur. This is illustrated in Scheme 1.21. Reaction A represents homodimerization of the reactive olefin; B the reaction of the resultant homodimer with the unreactive olefin; C the significantly slower homodimerization of the unreactive olefin; and D the reaction of the CM product with the unreactive olefin. C and D do not occur because they produce the less favoured

homodimer, and thus the primary product is the desired cross-metathesis product. It has actually been proposed that the reason for less-reactive olefins to not undergo homodimerization is their inability to form an alkylidene complex with ruthenium.



Scheme 1.21 – Metathesis reactions performed in the presence of a Grubbs catalyst. $R_1 \neq R_2$, Reactivity = $CH_2CHR_1 > CH_2CHR_2$

There are a large number of examples of successful cross metatheses in the literature; indeed, a good overview is given in the cross-metathesis chapter of the *Handbook of Metathesis*.[50] One particularly high-yielding example is given in Scheme 1.22.



Scheme 1.22 – CM between olefins of different reactivity

It has been suggested that α,β-unsaturated carbonyls are very slow to undergo homodimerization due to the instability of the α,β-unsaturated alkylidene intermediate,[51] shown in Figure 1.2.

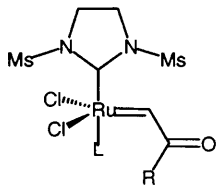


Figure 1.2 – α,β-unsaturated alkylidine

This is consistent with the finding that homodimerization of α,β-unsaturated amides requires a slightly modified version of **GII**[52] (Figure 1.3). The double bond presumably renders the *N*-heterocyclic carbene more electron rich, and this in turn can donate more to the ruthenium centre which affords the alkylidene intermediate greater stability.

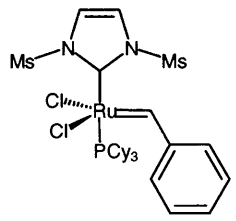


Figure 1.3 – Modified **GII**

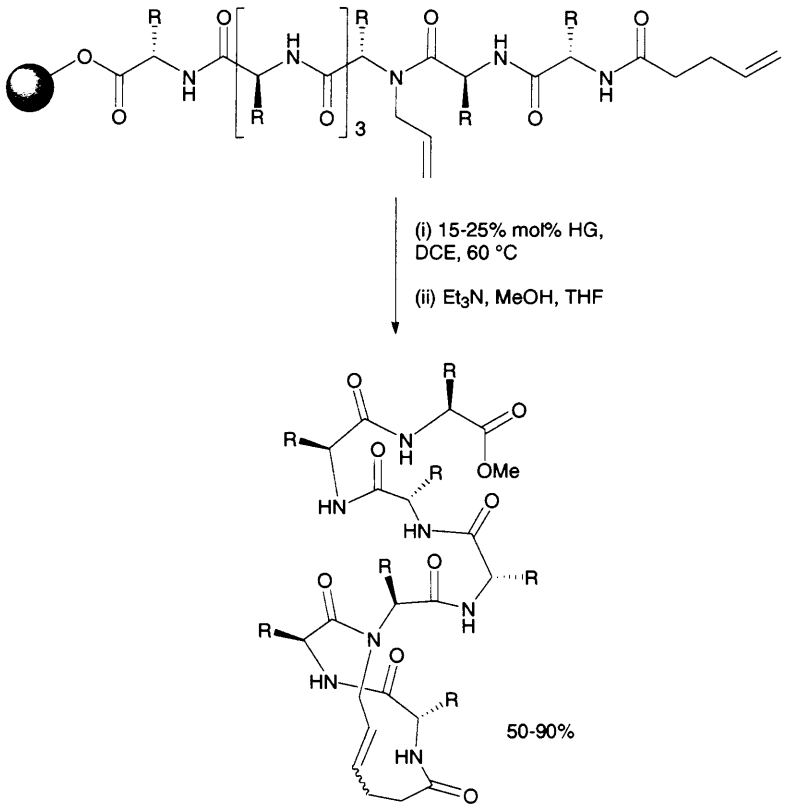
The further selectivity issue, common to all metathesis reactions, is *cis-trans* selectivity. In many cases of RCM reactions which produce five or six membered rings, only one geometry is possible. When ring sizes get much larger, however, the reaction often yields mixed geometries. In these cases, and for ROMP and CM reactions, the geometric outcome is dictated by thermodynamics. For example, cross-metathesis reactions involving initial loss of ethene afford products which continue interconverting, as long as they are in the presence of the catalyst. Therefore the thermodynamically favoured *trans* isomer is produced in greatest yield. Indeed in many cases of cross-metathesis the *trans* product is favoured, although often there is also a small accompanying yield of the *cis* product.

1.5 – Metathesis as a Tool in Chemical Biology

1.5.1 – Peptidomimetics via RCM

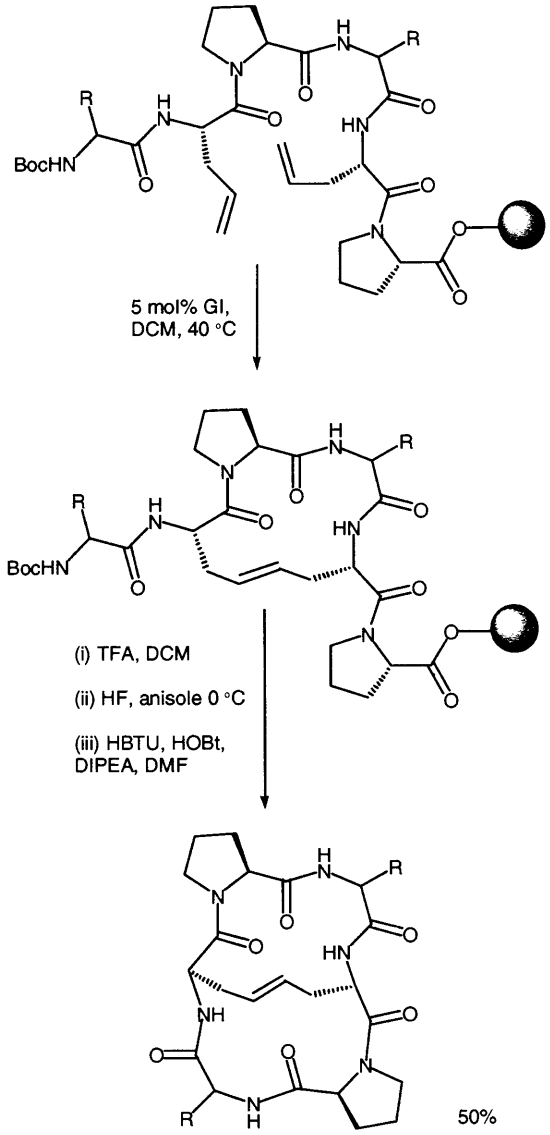
Perhaps of more relevance to this project are instances of metathesis used as a tool in peptide chemistry; and, indeed, such examples frequently occur in the literature. A few of the more relevant examples will now be considered. Often the metatheses are performed on-resin, a feature that makes the removal of ruthenium impurities facile.

Often the olefin moiety is used to afford a natural peptide greater metabolic stability by replacing a native feature such as a disulfide or hydrogen bond. RCM, with its entropic diving force, is an elegant means by which to achieve this. α-Helix mimics, for example, can be prepared by an on-resin RCM with **HG**[53] (Scheme 1.23). The precursors are simply synthesized using standard techniques and thus this may offer a simple route to robust α-helix analogues. This methodology has also been shown to be enhanced by use of microwave irradiation to effect the union of less reactive alkenes,[54] such as those bearing amino acids with *tert*-butyl protection.



Scheme 1.23 – α-Helix mimic prepared by RCM

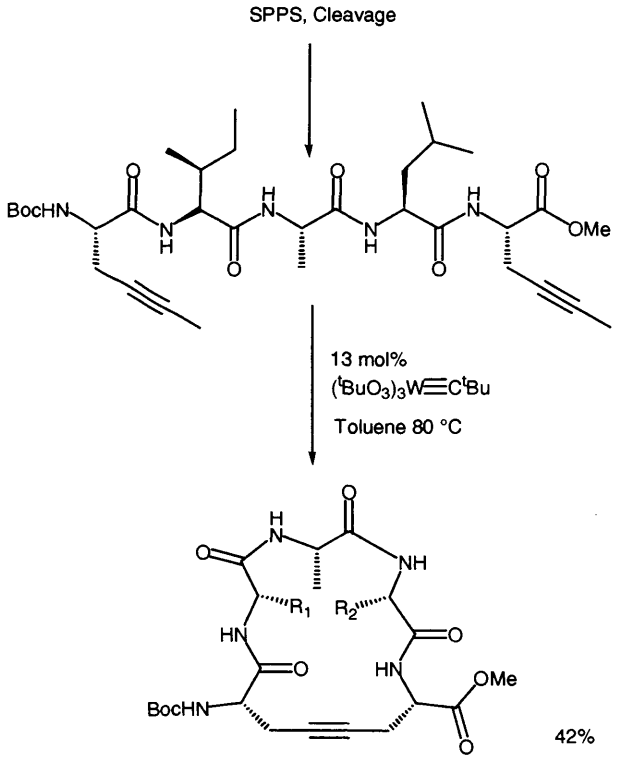
Additionally, RCM has been used in the preparation of cyclic peptides with improved biological properties.[55] Olefins are easily incorporated into peptide side-chains in the form of allyl glycine. The cyclic hexapeptide cyclo(D-Phe-L-Allgly-L-Pro-D-Phe-L-Allgly-L-Pro)cyclo(2-5) has been synthesized by an RCM with **GI** on resin prior to cleavage and cyclization (Scheme 1.24).



Scheme 1.24 – Synthesis of cyclic peptide analogues *via* RCM

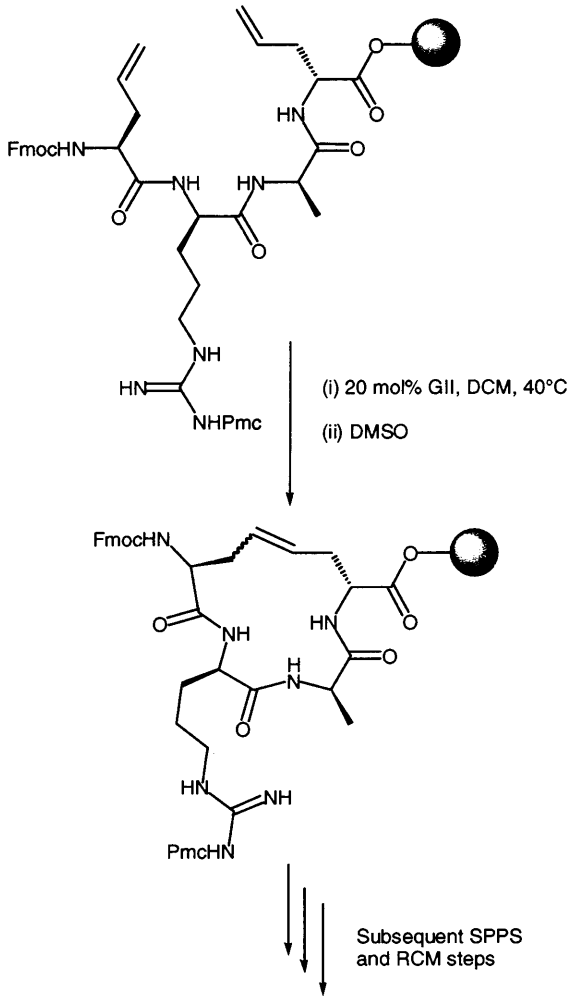
The bridging olefin affords the compound greater conformational restraint, a phenomenon that has been reported to result in a 10-100 fold increase in binding affinities of cyclohexapeptide Neurokinin A receptor antagonists upon introduction of a second ring *via* a lactam cyclization.[56] Additionally, the reaction is *trans* selective, presumably due to the conformational restriction afforded by the small ring structure. Disulfide bridges between cysteine residues can achieve this, but have lower metabolic stability. The replacement of sulfur-containing bridging groups with more metabolically

stable moieties *via* metathesis has also been used to synthesize modified portions of the lantibiotic nisin Z.[57] This was carried out using alkyne RCM with a tungsten catalyst to generate carbon-carbon triple bonds in place of sulfide bridges, in the solution phase. Scheme 1.25 shows the synthesis of one portion mimic as an example.



Scheme 1.25 – Synthesis of a portion of a Nisin Z analogue. $R_1 = C(CH_3)CH_2CH_3$; $R_2 = CH_2C(CH_3)_2$

More recently a modified lantibiotic was synthesized by solid phase peptide synthesis with three sequential RCM steps,[58] on resin with **GII** (Scheme 1.26). In this example, an alkenyl group replaces the thioether.

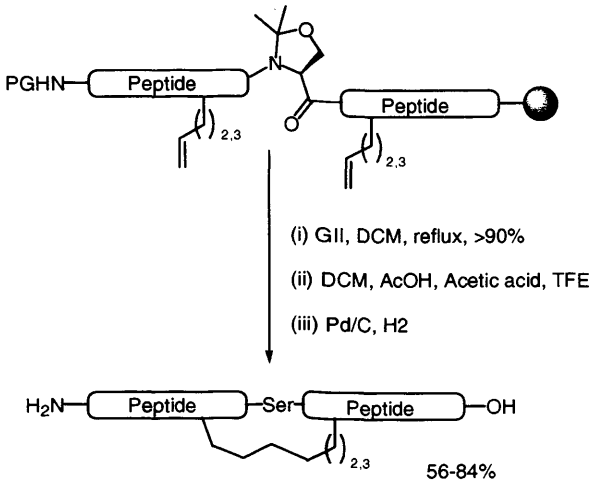


Scheme 1.26 – The first RCM in the synthesis of a tricyclic peptide. *E*/*Z* ratio not quoted

Cleavage yielded the desired analogue, however biological screening revealed the replacement of sulfide bridges with alkenes to be, in this case, deleterious. In contrast, when the disulfide bridge is replaced with an alkenyl group in the production of Oxytocin analogues,[59] the compound shows reduced, but still high, activity combined with a markedly increased half-life. This difference in activity is presumably due to the closer resemblance of the olefin moiety to the disulfide group than to the sulfide.

Noteworthy is the use of a similar on-resin RCM simply to produce a cyclic peptide. Schmiedeberg *et al.* report a synthesis of particular interest as it involves a cyclic 10-mer

involving no proline residues.[60] Until that time RCM-synthesized cyclic peptides always contained proline. Their poor yields could be significantly improved by introduction of a pseudoproline in a serine position (Scheme 1.27).



Scheme 1.27 – RCM in peptides containing no proline residues

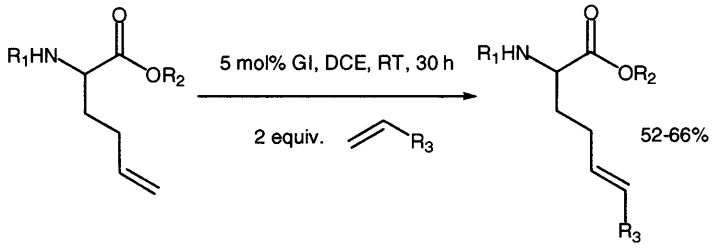
Solution phase RCM has been used to generate 16-membered peptidomimetics of the cyclic tetrapeptide Apicidin A in an analogous manner to equivalent on-resin cyclizations.[61]

These few examples highlight the applicability of metathesis with ruthenium catalysts in the presence of a wide range of functional groups. Increasing use of water soluble ruthenium precatalysts derived from functionalized **GII** to effect aqueous metathesis[62-64] indicates that the future of this area may well rely on aqueous-phase reactions and non-protected peptides. For this to work in peptide chemistry, cysteine and lysine residues will always require protection as they are known to poison Grubbs catalysts.

1.5.2 – Peptide Modification via CM

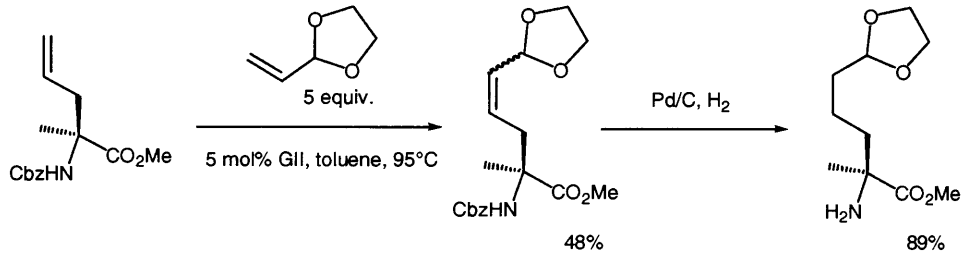
So far the examples we have seen have been RCM. We shall now turn to the applications of CM in peptide chemistry. CM was first used on protected amino acids by Gibson *et al.* using **GI**.[65] A number of differently protected homoallyl glycines were reacted, variously,

with styrene, hex-1-ene, and oct-1-ene, yielding in all cases homodimerization products in addition to the desired CM products in yields ranging from 52-66% (Scheme 1.28).



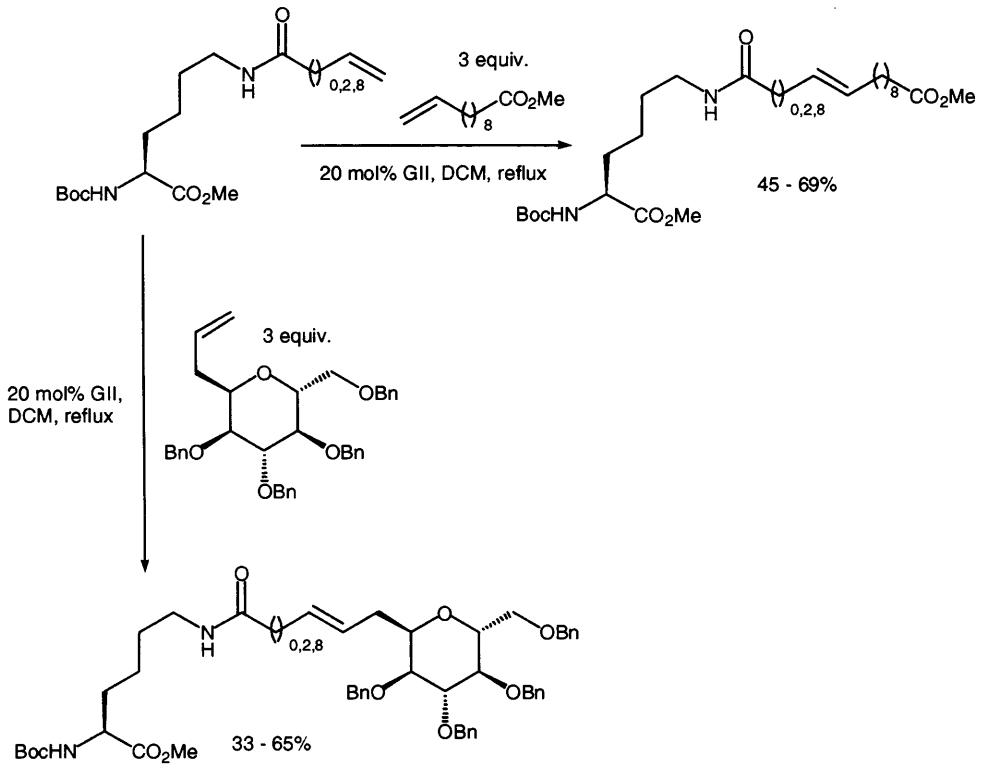
Scheme 1.28 – CM functionalization of homoallyl glycine. R_1 = Boc, Ac, Fmoc, Phth; R_2 = Me, Bn, $_t$Bu, H; R_3 = Ph, Bu, Hex.

This was not a particularly selective CM as the olefins have similar reactivity.[49] It was, however, an early demonstration of CM in the presence of Boc and Fmoc protecting groups and a free carboxylic acid. Far more recently CM has been use in the preparation of exotic α-substituted α-methyl amino acids in a similar side-chain metathesis reaction between olefinic compounds and an α-alkenyl side-chain on the α-methyl amino acid.[66] A typical example is given in Scheme 1.29.



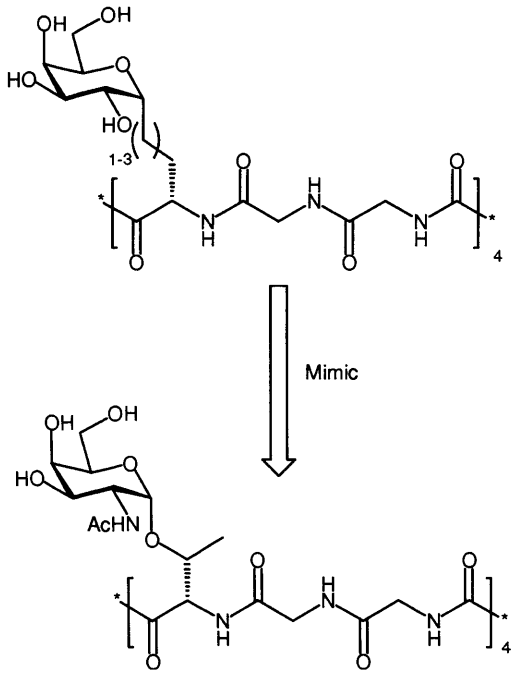
Scheme 1.29 – Synthesis of α-substituted α-methyl amino acids *via* CM

A logical development of such side-chain functionalizations would be the conjugation of amino acids to biomolecules such as sugars and fatty acids. Indeed Vernall *et al.* have achieved this using lysine and cysteine functionalized with an olefinic moiety, thus avoiding the use of expensive allyl and vinyl glycines.[67] These were reacted with a fatty acid and a glucoside, both bearing an olefinic group, yielding cross products and homodimers (Scheme 1.30). The homodimers of functionalized cysteine are potential candidates for replacement of disulfide bridges.



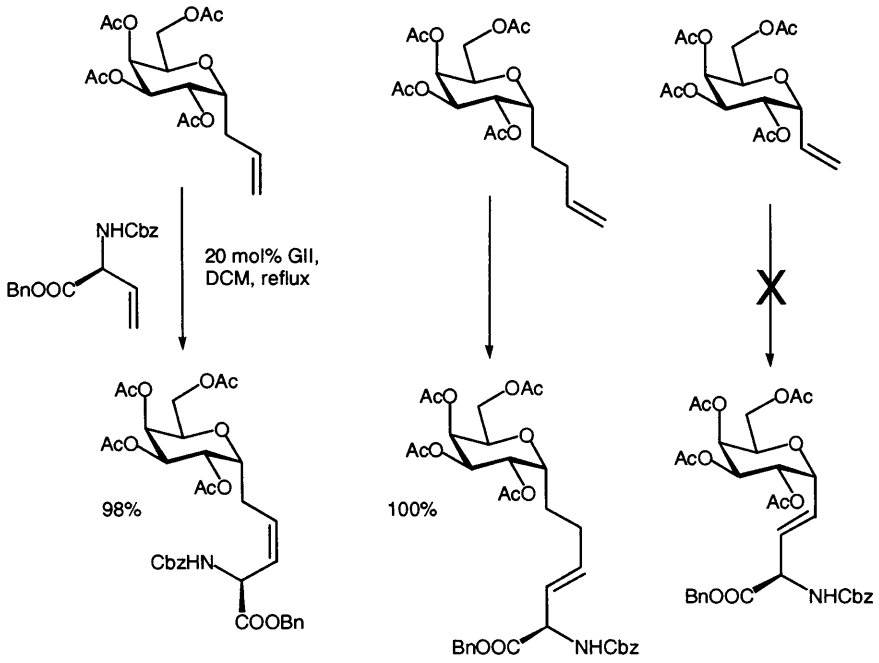
Scheme 1.30 – CM Functionalization lysine with a fatty acid or a glucoside

Some excellent CM selectivity in similar amino acid-sugar couplings was demonstrated in the synthesis of *C*-linked 'glyco' amino acids,[68] exotic building blocks for the synthesis of antifreeze glycoprotein analogues that ultimately replace a threonine derived ether linkage with an aliphatic carbon chain of variable length (Scheme 1.31).



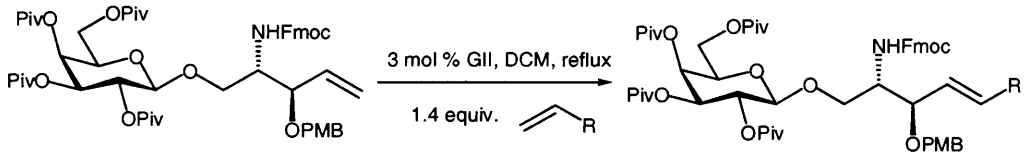
Scheme 1.31 – Saturated hydrocarbon mimic of glycosyl linkage

High yields were obtained for two metatheses (Scheme 1.32), however one proved problematic. It has been suggested that steric crowding is responsible for the lack of reaction in this example. In addition the oxygen atom in this case may chelate to the ruthenium, forming an inert metallocarbene, thus removing the catalyst from equilibrium. A more extensive study of CM generated glycosyl amino acids has dealt with selectivity in this reaction, and the results show that its success is dependant on the number of methylenes separating the olefin from both the glycoside and the amino acid.[69]



Scheme 1.32 – *C*-Linked glycoprotein analogues obtained by CM.

Various glycosphingolipid analogues have been synthesized by CM.[70] These proceed *via* a very selective CM using **GII** between an appropriate glycoside and various terminal olefins, the minimum yield being 76% (Scheme 1.33). It is worth highlighting that this reaction was an example of complete *trans* selectivity in most cases.



Scheme 1.33 – Synthesis of glycosphingolipids *via* CM. R = C_5H_{11}, C_7H_{15}, $C_{13}H_{27}$, $C_{15}H_{31}$

When a CM between the glycoside and an ethylene glycol derivative (Figure 1.4) was attempted, no product was obtained; the explanation given was the possible production of chelated metallocarbene intermediates.

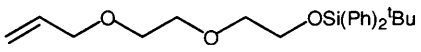
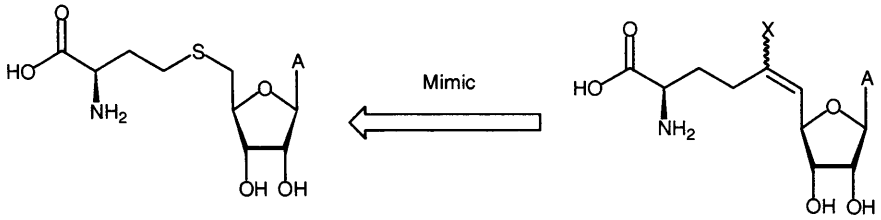


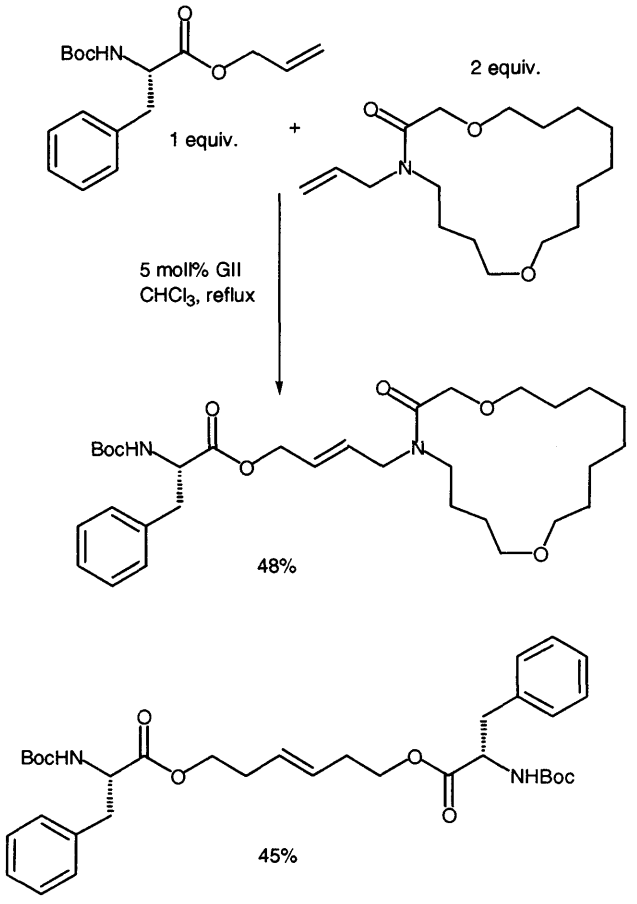
Figure 1.4

Interestingly, this problem has been overcome in the selective cross-metathesis between an adenosyl derivative and an olefinic six-carbon amino acid by switching to **HG**, affording impressive yields of 51%-77%.[71] This methodology seeks to prepare adenosylhomocysteine analogues and is, incidentally, another good example of the olefinic moiety replacing a sulfur-containing bridge (Scheme 1.34).



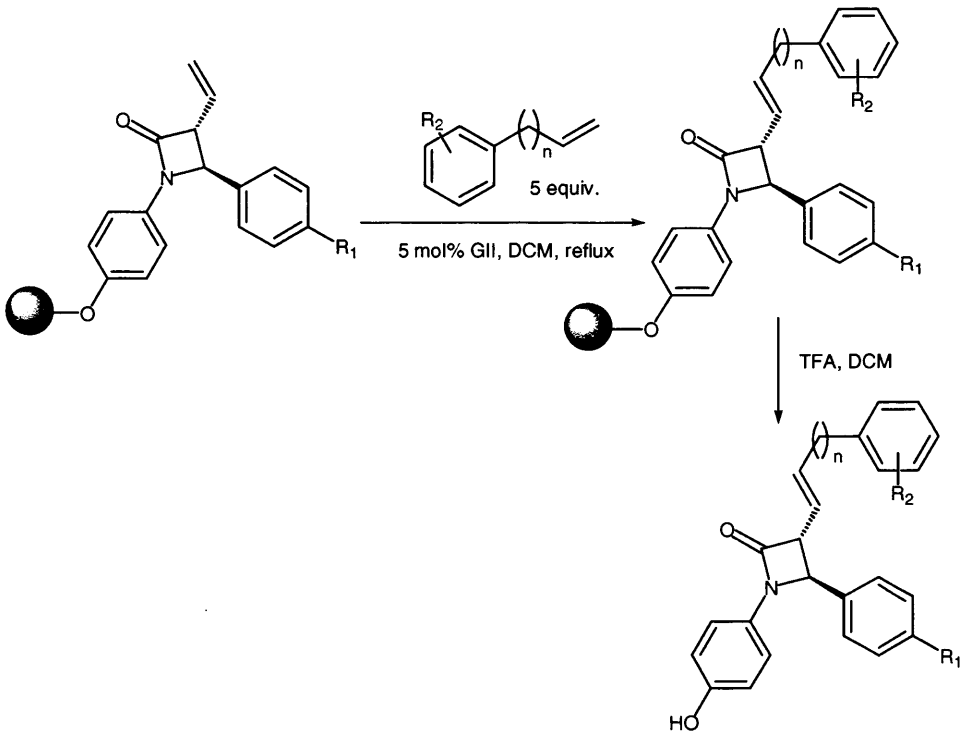
Scheme 1.34 – CM side-chain functionalization to mimic a sulfur-containing group.
A = Adenine

Continuing the discussion of CM couplings to amino acids, phenylalanine and a macrocyclic lactam have been joined *via* CM,[72] the idea being to develop the utility of macrocyclic lactams as scaffolds for peptidomimetics (Scheme 1.35). The CM product yield was only 48%, 45% of phenylalanine-derived material was isolated, however no macrocyclic lactam homodimer was observed. *Cis* : *trans* selectivity was 1:1.2. This example, despite not exhibiting great selectivity, highlights the potential of CM to generate interesting biologically inspired structures – the homodimer side products being of interest.



Scheme 1.35 – Synthesis of phenylalanine-macrocyclic lactam conjugate

On-resin CM has also been successful in the synthesis of functionalized β-lactams[73] (Scheme 1.36).



Scheme 1.36 – CM synthesis of functionalized β-lactams

Although this allows impurities to be simply washed away, in a typical CM five equivalents of non resin-bound olefin are used, and this is repeated a second time. The CM yields are fair (38-78% isolated), but this is only of any use if the excess olefin is cheap and readily available as ten equivalents are used. In this study the olefin was cheap, and the idea was to generate libraries of β-lactam derivatives.

In summary, metathesis, with its selectivity and mild reaction conditions, is a very good candidate for the modification of peptides and peptide mimetics. Indeed it has been widely used already, mostly for the generation of peptidomimetic structures by replacement of metabolically unstable structural features with non-natural structures which exhibit improved metabolic stability. Approaches appear to fall into two broad categories, RCM reactions which are typically performed on-resin, and CM functionalization of amino acids, which are more commonly used to provide a modified side chain.

1.6 – Crambin, a Small Hydrophobic Plant Protein

In order to investigate the utility of novel protocols for the chemical synthesis of proteins, the target protein should ideally be accessible by SPPS and well understood in terms of structure and folding. This allows for the effect of synthetic modifications to be effectively assessed. The plant protein Crambin is an ideal candidate.

Crambin is a hydrophobic protein 46 amino acids in length, first isolated from the seeds of the Abyssinian cabbage in 1965. Its primary structure has been determined by digestion and subsequent solid-phase Edmann degradation,[74] and it was found that it bore structural homology with plant toxins viscotoxin and purothionin. Crambin is non-toxic, possibly owing to its relative hydrophobicity, which may prevent it reaching the site of toxicity. Crambin could therefore be thought of as a 'cellular appendix,' although it is possible that its function has simply not been identified yet. It has also been established that Crambin exists in two forms in nature, one exhibiting Ser[22] and Ile[25] (SI form), the other Pro[22] and Leu[25] (PL form) (Figure 1.5).

SI form Crambin

Thr-Thr-Cys-Cys-Pro-Ser-Ile-Val-Ala-Arg-Ser-Asn-Phe-Asn-Val-Cys-Arg-Leu-Pro-Gly-Thr-**Ser**-Glu-Ala-**Ile**-Cys-Ala-Thr-Tyr-Thr-Gly-Cys-Ile-Ile-Ile-Pro-Gly-Ala-Thr-Cys-Pro-Gly-Asp-Tyr-Ala-Asn

PL form Crambin

Thr-Thr-Cys-Cys-Pro-Ser-Ile-Val-Ala-Arg-Ser-Asn-Phe-Asn-Val-Cys-Arg-Leu-Pro-Gly-Thr-**Pro**-Glu-Ala-**Leu**-Cys-Ala-Thr-Tyr-Thr-Gly-Cys-Ile-Ile-Ile-Pro-Gly-Ala-Thr-Cys-Pro-Gly-Asp-Tyr-Ala-Asn

Figure 1.5

These forms have been physically separated and crystal structures obtained,[75] showing the protein to include three disulfide bridges and consist of two α-helices and a β-sheet (Figure 1.6). Other regions constitute alternative turn-like structures.

Thr-Thr-Cys-Cys-Pro-Ser-Ile-Val-Ala-Arg-Ser-Asn-Phe-Asn-Val-Cys-Arg-Leu-Pro-Gly-Thr-Pro-Glu-Ala-Leu-Cys-Ala-Thr-Tyr-Thr-Gly-Cys-Ile-Ile-Ile-Pro-Gly-Ala-Thr-Cys-Pro-Gly-Asp-Tyr-Ala-Asn

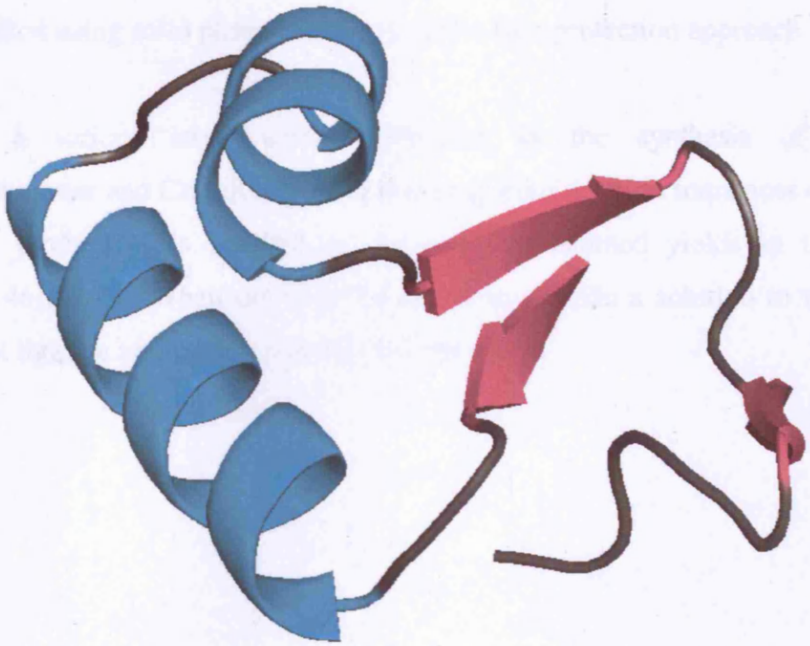
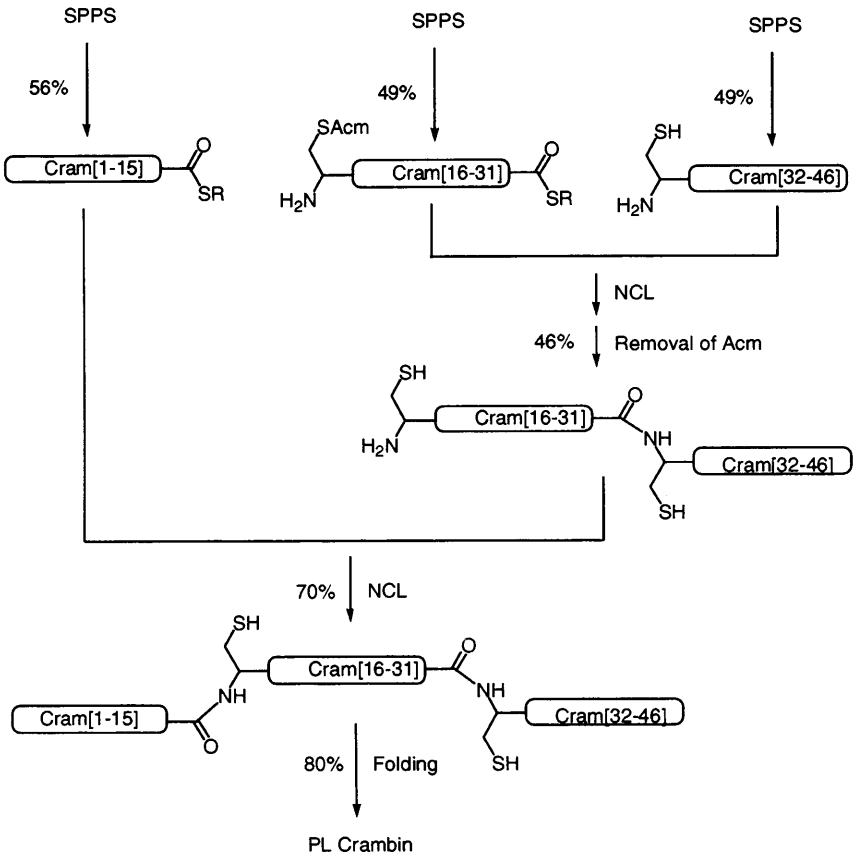


Figure 1.6 – The 3-D structure of Crambin and its amino acid sequence. Regions of α-helix are shown in blue; regions of β-sheet are shown in pink

The structure of Crambin has been probed by x-ray diffraction,[75-77] NMR,[78-81] circular dichroism,[82, 83] Raman spectroscopy[84] and sulfur atom scattering.[85] Molecular dynamics[86] and Monte-Carlo techniques[87] have been employed to study its folding. Data obtained from a synthetic Crambin analogue could therefore be compared to the extensive literature, and it would thus be possible to accurately determine the effect the inclusion of the non-natural linker has on its structure.

Two chemical syntheses have been reported, both by the Kent group, and are especially worthy of discussion as they provide excellent examples of the use of NCL and related strategies. The first approach initially sought to prepare the protein by NCL between two peptide segments.[88] Two strategies were investigated, ligation between the Cram[1-31]$^{\alpha}$thioester and Cram[Cys32-46] in the synthesis of the SI form; and between the Cram[1-15]$^{\alpha}$thioester and Cram[Cys16-46] in the case of the PL form. Residue 15 happens to be valine and so, due to the anticipated difficulty of achieving NCL at a Val-Cys junction, the sterically encumbered valine was substituted for alanine. Peptides were synthesized using solid phase techniques and a Boc-protection approach.

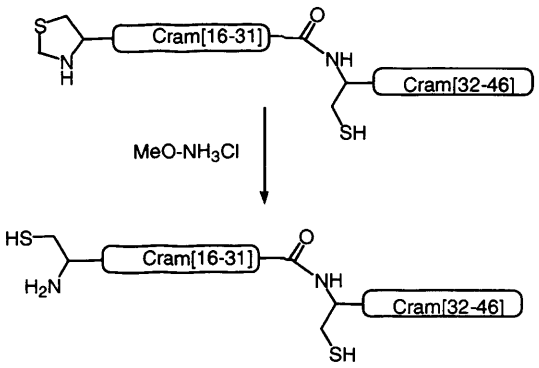
There was a serious issue with purification in the synthesis of both the Cram[1-31]$^{\alpha}$thioester and Cram[Cys16-46] resulting from deletion sequences eluting very close to the product. This resulted in unfavourable isolated yields in the case of Cram[Cys16-46], 4.7%. When optimization failed to provide a solution to this issue, a three segment ligation approach was used (Scheme 1.37).



Scheme 1.37 – 3-segment approach to the chemical synthesis of Crambin

The linear peptide was characterised by electrospray MS, and after folding the mass change was consistent with the formation of disulfide bonds. Circular dichroism and ^{1}H NMR data were compared with the literature and thus the structure was confirmed to be identical to that of Crambin isolated from the Abyssinian cabbage. The overall yield was ≈25%.

The second synthesis built upon this methodology in the development of a one-pot total synthesis of Crambin.[89] Use of the Acm protecting group on the Cram[16-31] segment is problematic in a one-pot approach as the second NCL works significantly better when the deprotected Cram[16-46] is purified. Replacement of Cys[16] with the 1,3-thiazolidine-4-carboxo group allowed for generation of the N-terminal cysteine and resulted in a crude mixture amenable to the second ligation, and thus potential for use in a one-pot approach (Scheme 1.38).



Scheme 1.38 – Alternative cysteine-protection approach

The perfected one-pot protocol allowed for generation of Crambin in an improved overall yield of ≈40%, in less than a quarter of the time taken for the previous synthesis.

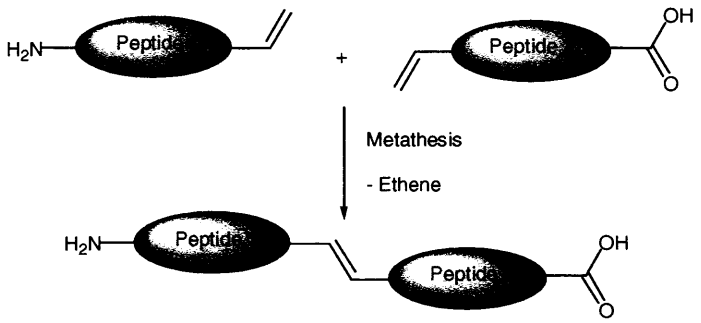
In conclusion, Crambin is an excellent choice for investigating the applicability of novel synthetic peptide-modification protocols. Its structure has been extensively studied and has a small size, and so the SPPS steps promise to be reasonably straightforward. Indeed work synthesizing Crambin by the Kent group using a Boc-protection approach has been fruitful.

Chapter 2 – Novel Synthetic Ligation – Results and Discussion

2.1 – Introduction

2.1.1 – Overview

The main aim of the project was to examine the possibility of using metathesis to ligate two peptide fragments in a 'semisynthetic' manner, analogous to the various reported ligation methods described in Chapter 1 (Scheme 2.1).



Scheme 2.1 – Proposed ligation method based on metathesis

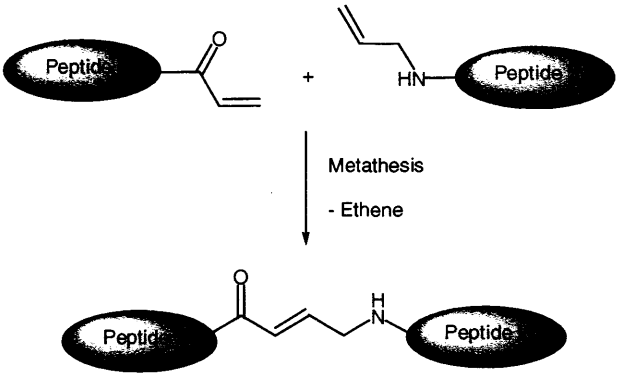
The metathesis reaction is attractive in this context due to its high functional group tolerance and, as has been discussed, it has been used for a number of applications in the field of peptide chemistry. Furthermore, native peptides possess no olefinic groups. As any ligation strategy relying on metathesis would generate an olefin, the potential would exist for site-specific attachment of one of any number of functional motifs to a protein *via* an olefin-specific reaction. Thus the application of metathesis to the ligation of peptide fragments in the synthesis of proteins would allow for manipulation of their finely tuned biological specificity in the development of new functional materials.

The success of metathesis as a means of peptide ligation relied on two important factors: the viability of olefin incorporation at peptide *C*- and *N*-termini, and chemoselectivity in the CM between these olefin-functionalized peptides. Peptide chemistry is very costly, and therefore it is imperative that the reaction is as chemoselective as possible, minimising wasteful homodimerization and maintaining the inherent directionality of peptides. At this juncture it is worth acknowledging that peptide homodimerization

products have potential in areas such as the synthesis of unnatural functional peptides, but as they will have lost the *N*→*C* directionality, they are therefore not of interest for protein semisynthesis, and thus this discussion.

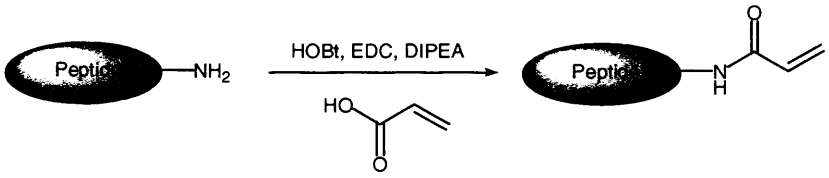
2.1.2 – Selection of Appropriate Olefins for Initial Investigation

The selectivity rules defined by Chatterjee *et al.*[49] are based upon the ability of olefins to undergo homodimerization, as described in Section 1.4.3. α,β-Unsaturated carbonyls are electron poor and hence undergo homodimerization sufficiently slowly to be considered as good partners with, for example, allylamines. Thus if two different peptides could be functionalized with these groups, one could expect a CM between them to be chemoselective (Scheme 2.2).



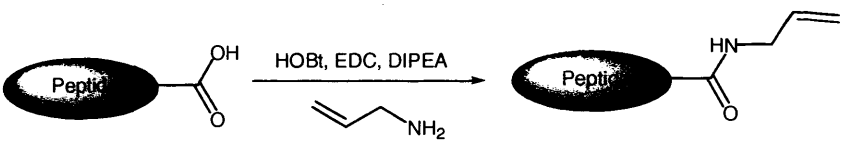
Scheme 2.2 – Chemoselective cross metathesis of peptides

Access to the α,β-unsaturated carbonyl functionalized peptide promised to be compatible with peptide chemistry, inasmuch as standard peptide coupling conditions with acrylic acid could potentially be used (Scheme 2.3).



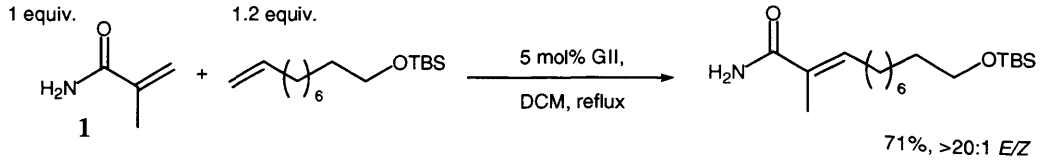
Scheme 2.3 – Functionalization of a peptide *N*-terminus with acrylic acid

This, of course, would generate an acrylamide, which is a type II olefin, at the *N*-terminus of a peptide. At this point, the easiest way to functionalize the *C*-terminus with a more reactive type I olefin would be to couple it with allylamine using standard peptide coupling conditions (Scheme 2.4).



Scheme 2.4 – Functionalization of a peptide *C*-terminus with allyl amine

The report by Chatterjee *et al.*[49] of an efficient cross metathesis of acrylamide **1** and a terminal olefin moiety (Scheme 2.5) provided good evidence to support the proposed cross metathesis. It is important to note that this reaction employed a small excess of the type I olefin and yet proceeded in good yield and with good selectivity. These are important factors for application of this type of methodology to peptide and protein synthesis.



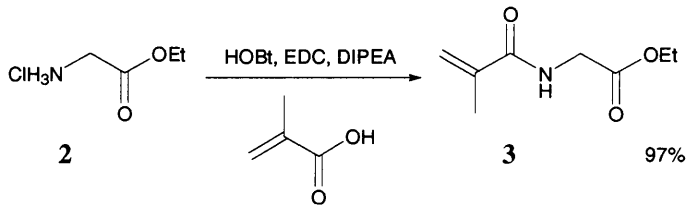
Scheme 2.5 – Selective CM involving an acrylamido group[49]

2.2 – Establishment of a CM Ligation Protocol

2.2.1 – Attempt using an Allyl Amine Functionalized Substrate

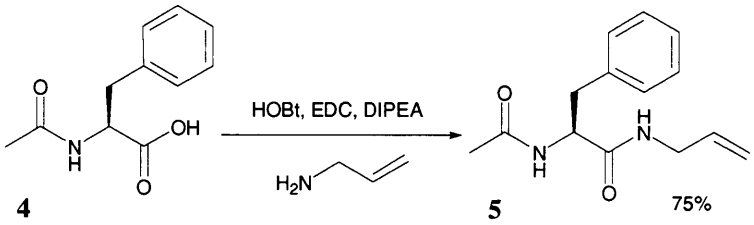
Initially the establishment of a successful protocol for metathesis employing single amino acids was sought. Such an approach would require appropriate protecting groups at either the *C*-terminus or the *N*-terminus, depending on which position was to be functionalized. *N*-acyl protected amino-acids and *C*-terminal esters were utilised as protecting groups.

The amino acid chosen for *N*-functionalization was glycine ethyl ester **2**, and in order to emulate as closely as possible the structure of acrylamide **1** in the above example (Scheme 2.4), methacrylic acid was used. Functionalization proceeded smoothly giving the required derivative **3** in an excellent 97% yield (Scheme 2.6).



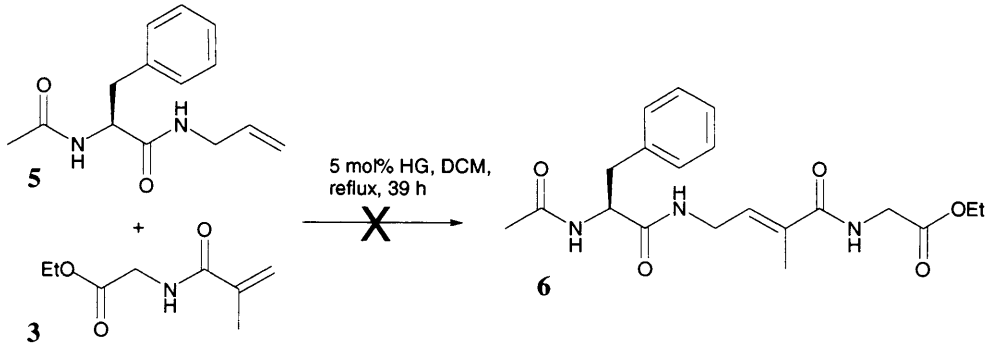
Scheme 2.6 – Synthesis of **3**

Similar conditions were used in the functionalization of *N*-acyl phenylalanine (**4**) with allylamine to provide a partner for CM, giving **5** in 75% yield (Scheme 2.7).



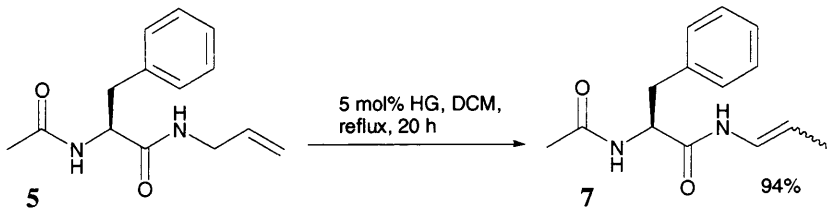
Scheme 2.7 – Synthesis of **5**

CM was now attempted between **3** and **5** (Scheme 2.8). The initial conditions investigated were based on those frequently reported in the literature;[50] 5 mole per cent **HG** was used in refluxing DCM.



Scheme 2.8 – Attempted CM between **3** and **5**

Unfortunately, **6** was not isolated; starting material **3** was recovered impure, and the isomerised product **7** was obtained in 94% (Scheme 2.9) as an inseparable mixture of geometric isomers (*Cis*: *trans*, 22:25).



Scheme 2.9 – Rearrangement of allyl amide moiety. *Cis*: *trans*, 22:25

This phenomenon had been reported[45] and this type of ruthenium catalysed isomerisation has been used beneficially in certain studies.[90, 91] Moreover, Steinke and co-workers had studied the effect of various additives on the isomerisation, finding that monophenyl phosphoester (Figure 2.1) can act as a suppressant.[92] An investigation into the application of this modified protocol was considered, however it was decided to vary the olefinic amine instead, and thus hopefully avoid this rearrangement process.

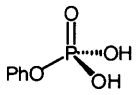
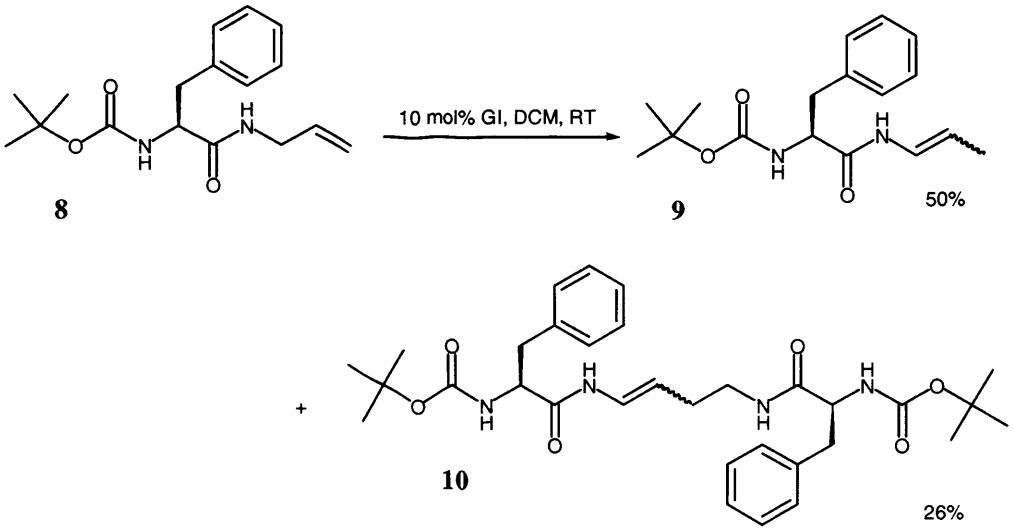


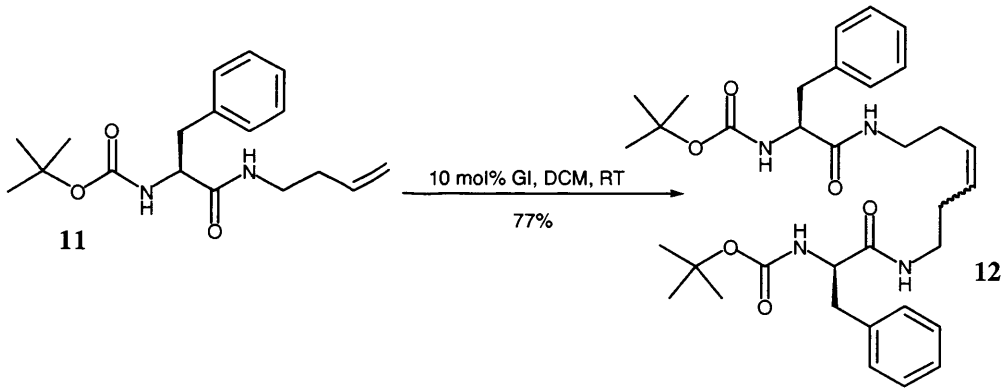
Figure 2.1 – Monophenyl phosphoester

More recently a paper had reported a similar rearrangement the allyl amide **8** in a study on the homodimerization of amino-acid allyl- and homoallyl-amides using 10 mol% **GI** in DCM at RT (Scheme 2.10).[93]



Scheme 2.10 – Attempted homodimerization of BocPhe allyl amine derivative by Miller *et al*

The paper also reports the homodimerization of homoallyl amide **11** (Scheme 2.11) which apparently proceeds without rearrangement.



Scheme 2.11 – Homodimerization of homoallyl amine derivative by Miller *et al*

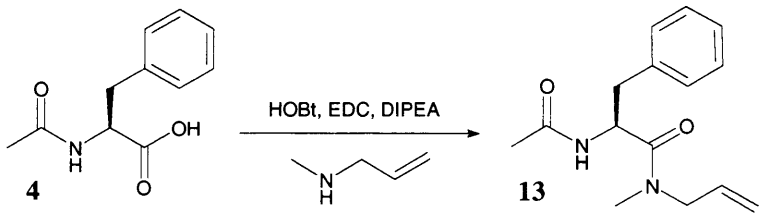
Homoallylamine-functionalized substrates were an attractive prospect as rearrangement in subsequent metathesis would be avoided, but we decided first to investigate an *N*-methyl allylamine functionalized substrate.

2.2.2 – Attempts using an N-Methyl Allylamine Functionalized Substrate

Metathesis reactions involving *N*-protected allyl amides have been described in the literature and of particular relevance were the RCM reactions of *N*-methyl allyl amides. An example is given in Scheme 2.12.[94-96]

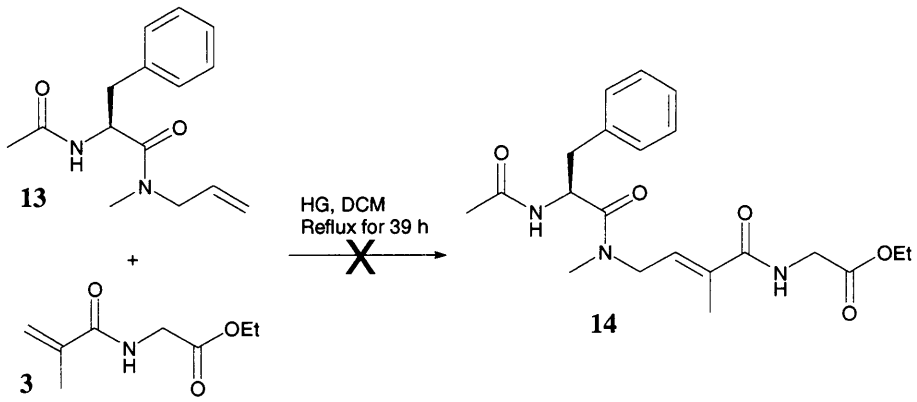
Scheme 2.12 – RCM of an *N*-methyl allyl amide [80]

Moreover, an example of successful CM of a *N*-methyl allyl carbamate has been reported.[97] CM of the analogous *N*-methyl substrate therefore appeared worthy of investigation and so **4** was functionalized with *N*-methyl allylamine yielding **13** in 86% yield (Scheme 2.13).



Scheme 2.13 – Synthesis of **13**

The CM between **3** and **13** was now attempted using a variety of conditions; however no identifiable products were isolated (Scheme 2.14).



Scheme 2.14 – Attempted CM between **3** and **13**

Initially, the metathesis utilized 20 mol% **HG**. After refluxing in DCM for 39 h, starting materials still appeared to be present. Separation of the resultant compounds and NMR analysis indicated that neither the cross metathesis nor the homodimerization of **13** had occurred. A substantial amount of **3** was recovered in an impure form. NMR spectra of all other isolated materials could not be assigned, and were not consistent with a CM product. Unfortunately this was the case with all subsequent attempts to generate **14**. These investigated the effect of higher temperatures using refluxing toluene and microwave irradiation, and the use of **GII** in place of **HG**. Furthermore, simple homodimerizations of **13** were attempted, with no success.

It was clear that **13** underwent no desired metathesis under these various conditions. It is possible that one of the ruthenium-**13** Alkylidene complexes **15** or **16** is unstable due to steric constraints resulting from the *N*-methyl group (Figure 2.2).

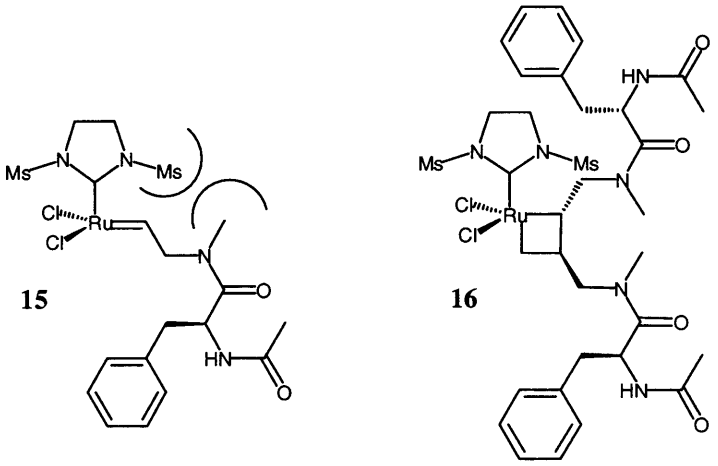


Figure 2.2 – Alkylidene intermediates **15** and **16**

This explanation is supported by the fact that that *N*-methyl allyl amides have been shown to undergo metathesis,[94-96] and yet no such homodimerizations have been reported. Our inability to obtain the homodimer in the absence of **3** was further indicative of this argument. In conclusion, a different olefinic amine needed to be used for *C*-terminal olefin-incorporation.

2.2.3 – Peptidomimetic Considerations

Potential structural implications of introducing metathesis-derived residual atoms into a peptide main-chain had to be considered. The product of a successful CM reaction between allylamine functionalized AcPhe, **5**, or its methylated analogue, **13**, with **3** would comprise a resultant linker one less carbon in length than two amino acid residues (Figure 2.3).

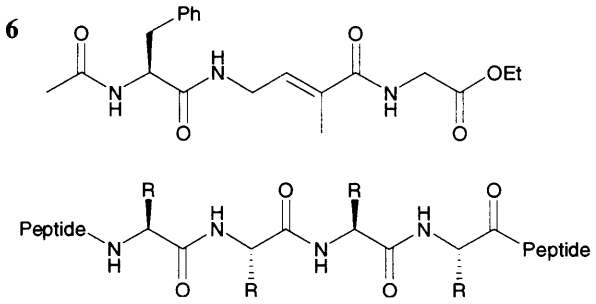
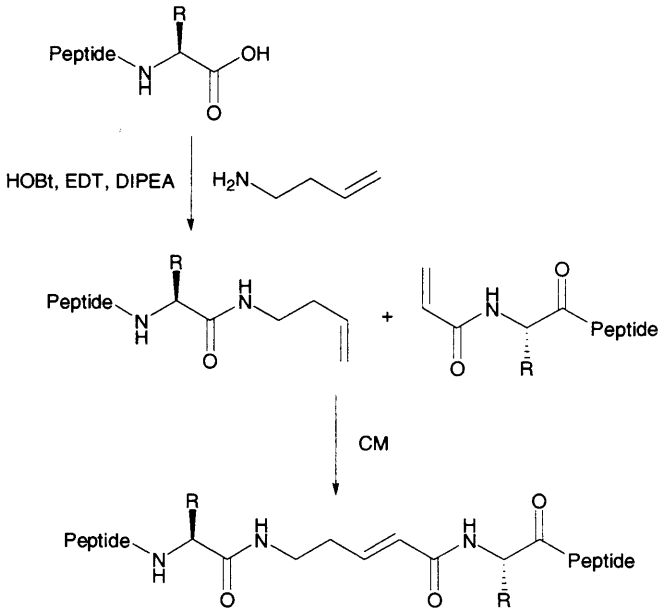


Figure 2.3. – CM product **6** and native peptide. R = variable side chain.

If the ultimate goal of this methodology is to synthesize functioning peptides, a residual group one atom less than the length of two amino acids is potentially unfavourable. This is because there is a high probability that lengthening the amino acid chain will have a profound effect on the folding of the protein and thus an adverse effect on its function. Notwithstanding this, we were hoping such site-specific manipulation of main-chain length would provide a valuable tool for structural studies of proteins.

However, from the perspective of the development of a methodology for the ligation of peptides in order to produce functional peptides, as opposed to study structure and folding, the inclusion of a non-natural resultant linker was a potentially serious problem with the methodology. At this point it was decided to pursue a strategy which resulted in the replacement of two amino acids. The site of replacement, naturally, would require careful consideration so that this replacement itself has minimal effect on the folding of the peptide.

In light of this new approach, we were keen to investigate homoallylamine as a means of *C*-terminal olefin-functionalization. The inclusion of the additional CH_2 group would yield a linker with the same number of bonds as two amino acids in the CM product (Scheme 2.15).



Scheme 2.15 – Proposed use of homoallyl amine in peptide ligation

The linker in the product molecule is a good candidate for a peptidomimetic of two amino acids – it has the same length* and lacks a single amide bond (Figure 2.4). We were further encouraged to see very similar peptidomimetic structures in the literature (see below).[98-101]

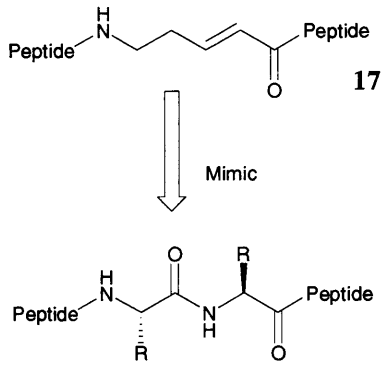


Figure 2.4 – Synthetic peptide mimic **17**

This methodology therefore had the potential to provide access to much larger peptides, introduce a site bearing an olefin moiety which could be subsequently functionalized *and* selectively remove a single amide bond from a peptide chain. This third possibility was particularly interesting given recent work at the time by Kelly, who had used the Wittig reaction to introduce a *trans* carbon-carbon double bond into the peptide chain, replacing an amide bond.[98] This approach was attractive as it mimicked the conformationally constrained amide bond by exact substitution with the olefin (Figure 2.5). They went on to show how removal of H-bonding ability in a single site of the Ab peptide inhibited the formation of fibrils or protofibrils in amyloidogenesis. More recently this inhibition effect has been compared to the corresponding ester peptidomimetic, and their toxicities investigated.[102]

* Length of standard dipeptide is 7.27 Å. The predicted length of linker based on measured bond lengths of simple organic molecules is 7.33 Å. Standard bond lengths in peptide (Å) – C_α-C, 1.51; C-N, 1.32; N-C_α, 1.46. C=C bond length is 1.34 (ethene).

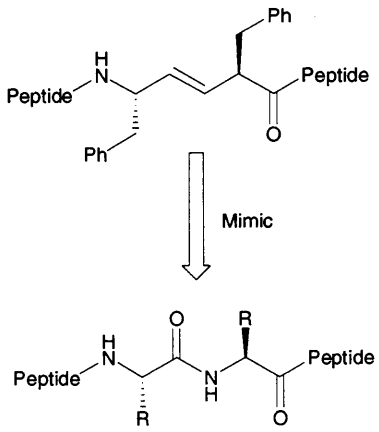


Figure 2.5 – Peptidomimetic reported by Kelly

Z-Fluoroalkene dipeptide isosteres are another example of a peptidomimetic in which there is exact substitution of an amide bond for a carbon-carbon double bond (Figure 2.6).[99, 100]

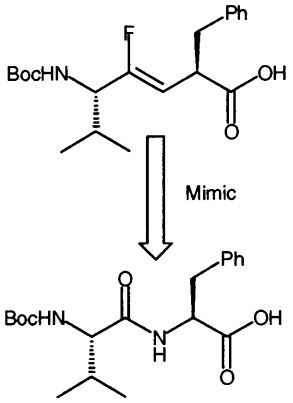


Figure 2.6 – *Z*-fluoroalkene dipeptide isostere[99]

The fluorine atom forms hydrogen-bonds in a similar manner to the carbonyl oxygen in the native structure, and so these structures potentially allow for a more faithful level of mimicry. The synthesis of GPR-54-agonistic pentapeptide analogue exhibiting a *cis*-amide mimetic *E*-fluoroalkene moiety is a good example of the utility of such structures.[101] In this study the analogue was shown to have significantly decreased activity, and thus the importance of a specific *trans* amide bond in the native agonist could be inferred. The relationship between this isostere and the *cis* and *trans* amide bonds is shown in Figure 2.7.

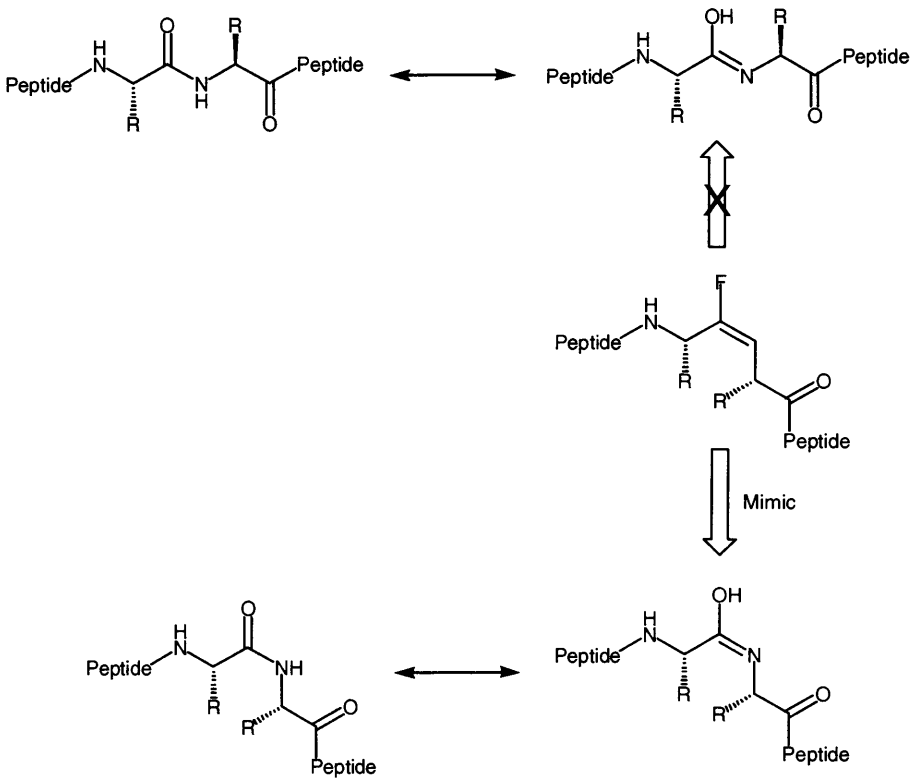


Figure 2.7 – Relationship between the *E*-fluoroalkene and the amide bond

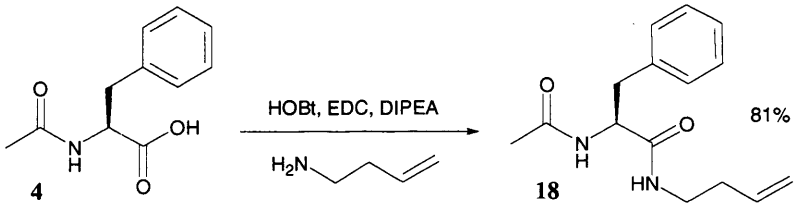
If our CM approach were to be used to generate peptidomimetic **17**, it is worth noting that the inserted double bond would be shifted with respect to the native amide bond, in contrast to these examples. Furthermore the CM approach would yield no side-chain functionality. Our methodology however, by virtue of its convergent nature, allows for potential access to larger peptides than these examples, in which the modified portion is introduced into the peptide chain *via* simple linear SPPS techniques and are thus subject to their inherent restrictions.

Overall, the potential of the linker in **17** (Figure 2.3) to be used as a peptidomimetic provided a firm incentive to pursue an approach that utilized homoallyl amine in favour of investigating other classes of linkage or rearrangement-suppressing additives as described by Steinke *et al.*

2.2.4 – Successful CM using a Homoallyl Amine Functionalized Substrate

2.2.4.1 – Ligation of AcPhe and PheOEt

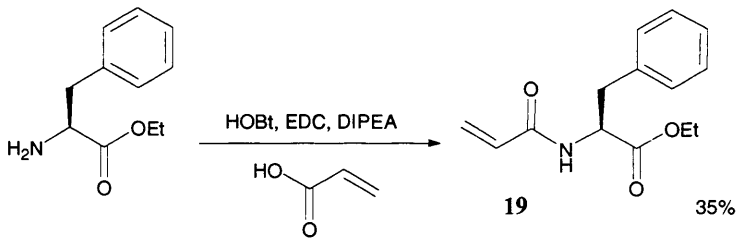
The CM of homoallylamine functionalized substrates was examined. Homodimerization of *C*-homoallyl amide Boc amino-acids using **GI** has been reported,[93] and does not suffer from rearrangement problems that have previously been discussed. Given that homodimerization is a key step in CM,[49] this was very encouraging. Furthermore we noted an example of successful RCM involving a homoallyl amide in the literature.[103] Of note was the ease of synthesis of the precursor, in this case **4** was functionalized with homoallylamine, generating **18** in 81% (Scheme 2.16).



4 **18**

Scheme 2.16 – Synthesis of **18**

In addition to this a new *N*-acryloyl amino acid, **19**, was prepared in 35% yield by subjecting phenylalanine ethyl ester hydrochloride to acrylic acid in a standard peptide coupling (Scheme 2.17).

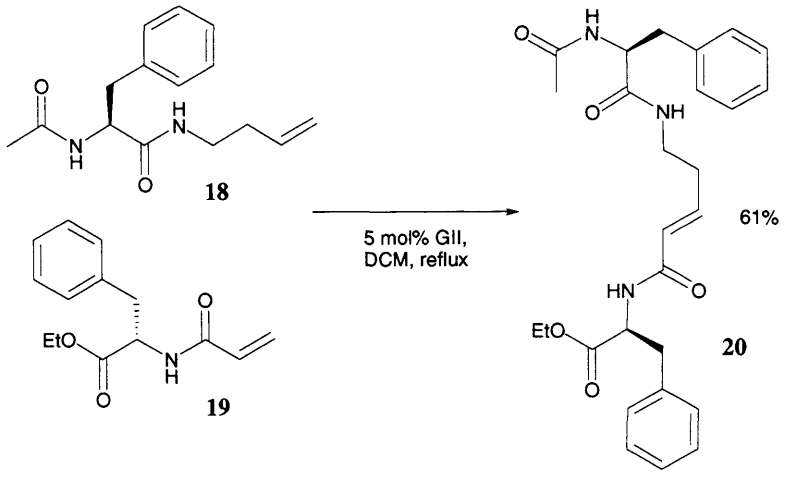


19

Scheme 2.17 – Synthesis of **19**

Acrylic acid was chosen in place of methacrylic acid as it would simplify the resultant linker unit. From a peptidomimetic standpoint the methyl group in compound **3** was undesirable. Furthermore some excellent CM yields of acrylamides have been reported by the Grubbs group.[104]

18 and **19** were reacted in a 1:1 ratio, in refluxing DCM, with 5 mol% **GII**, and after 24 h a white precipitate had formed. The reaction mixture was purified directly using column chromatography on silica gel, yielding the CM product **20** in 61% (Scheme 2.18). This simple workup was used for all subsequent CM reactions.

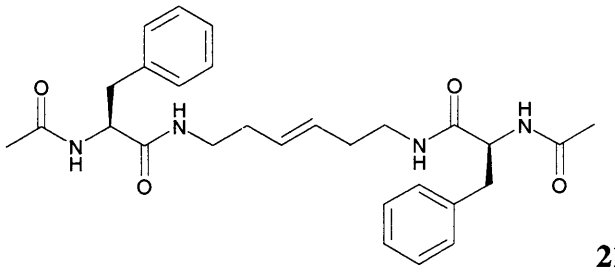


Scheme 2.18 – First successful CM

We were pleased to observe that the product was obtained as a single isomer, exhibiting a vicinal coupling of 15 Hz between olefinic protons, confirming it to be a *trans* double bond. In terms of peptidomimetics this was ideal as it installed a structural motif with a conformation closer to that of the native peptide than in the case of the *cis* isomer.

The reaction was repeated using a 1.2 equivalent excess of **18**, and 7 mol% of **GII**, yielding an excellent 90% CM product. Additionally, in this case, a 15% yield[†] of homodimer **21** was isolated (Figure 2.8), supporting the proposed CM mechanism, i.e. the reactive olefin dimerizing first, prior to reaction with the unreactive olefin. It must be stressed now, however, that homodimers were generally not isolated.

[†] Calculated from 1.2 equivalent excess of **18**



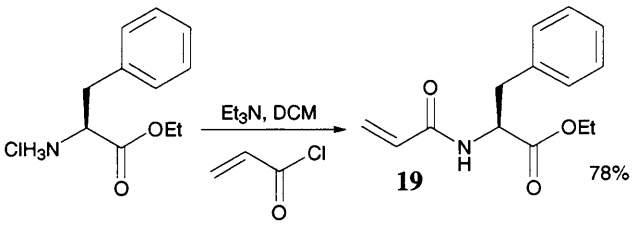
21

Figure 2.8 – Isolated homodimer

2.2.4.2 – Synthesis of Additional Acryloyl Amino-Acid Species

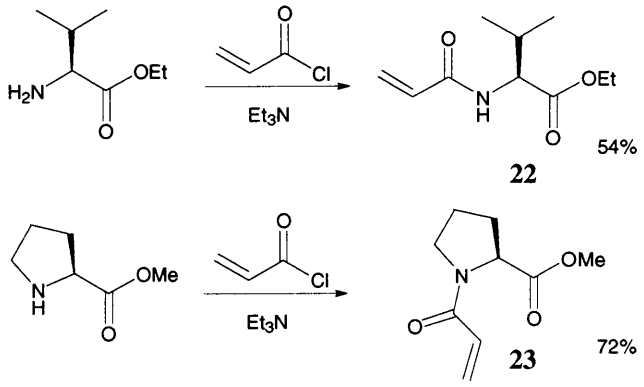
In order to demonstrate the applicability of this reaction to the ligation of various amino acids, it was decided to generate a range of *N*-acryloyl amino acid esters and attempt the CM of each with **18**. The coupling of amino-acid esters to acrylic acid under peptide coupling conditions proved a very poor method of acryloylation, indeed serine methyl ester and cysteine ethyl ester could not be functionalized in this manner.

A paper by Bentolila *et al.* reports the use of 1.1 equivalents of acryloyl chloride, a commercial, stable reagent, as a means to accessing *N*-acryloyl amino acids.[105] It allowed simpler, more economical access to **19** for further studies (Scheme 2.19).



Scheme 2.19 – Improved synthesis of **19**

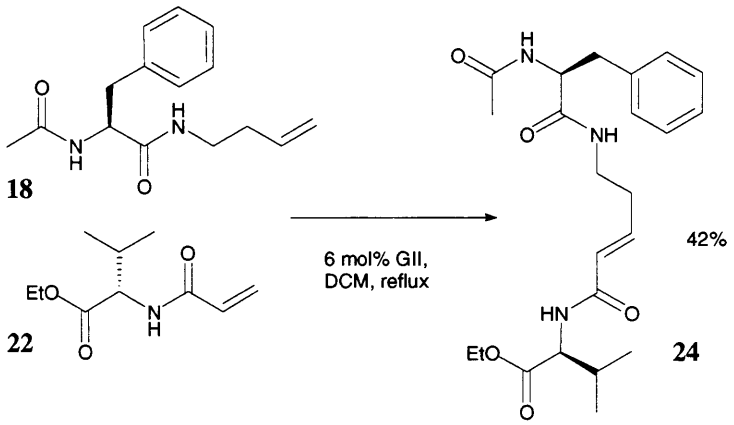
Esters of valine and proline were functionalized in a similar manner to phenylalanine ethyl ester generating **22** and **23**, in yields of 54% and 72%, respectively (Scheme 2.20).



Scheme 2.20 – Synthesis of olefin substrates **22** and **23**

2.2.4.3 – Ligation of AcPhe and ValOEt

CM between **18** and **22** was performed in refluxing DCM (Scheme 2.21). This reaction used a 1.1 excess of **18** and 6 mol% **GII** and yielded 42% **24** after 24 h.



Scheme 2.21 – CM between **18** and **21**

TLC analysis of limiting reagent, **22**, was used to monitor the reaction and after 24 h it appeared that a small quantity of **22** persisted. This was attributed to the equilibrium between **22** and the starting material due to a small amount of ethene remaining in solution, and so the reaction was terminated. The CM was repeated using 10 mol% of **GII**. After 72 h **22** was still apparent by TLC, but the reaction was terminated and an improved yield of 55% was obtained. One possible explanation is that the catalyst has a limited lifespan under these conditions, and hence the prolonged reaction time was not beneficial *i.e.* the increase in yield could be solely attributed to the additional catalyst.

The lower general yield may be tentatively attributed to the steric bulk of the valine side chain, as this may lead to a high energetic barrier to complexation to the ruthenium prior to metallocycle formation. We noted that one of the requirements for optimal NCL of peptides is lack of β-branching in the *C*-terminal amino acid.[17, 33] Therefore such issues associated with the ligation of β-branched amino acids were anticipated.

At this juncture, two successful CM ligation reactions had been achieved using homoallyl amine- and acryloyl chloride-functionalized amino-acids. This approach allows for the introduction of a dipeptide isostere in addition to effecting peptide ligation. We were keen to perform the reaction in the presence of a range of functional groups, and thus demonstrate a wider scope.

2.3 – Demonstration of the Stereochemical Integrity of Ligated Amino-Acids

Given the initial success of the CM protocol, it was imperative to corroborate the stereochemical outcome of these reactions. Stereochemistry is an important factor in biological systems and if this methodology is to be applied to generating materials to probe biological systems then stereochemical integrity is likely to be an important factor. If the stereochemistry of one amino acid in a protein is inverted, it could result in loss of function, change of folding pattern, or, at the other extreme, it could have no observable effect. This provided a firm incentive to establish the conservation of stereochemistry throughout every class of reaction in the protocol.

2.3.1 – The Issues Associated with Polarimetry

Polarimetry provides information with no use in the assignment of structure, or even the determination of whether or not any degree of racemization has occurred – a zero measurement does not necessarily mean racemization has occurred, and any non-zero reading could result from any non-equal ratio of enantiomers. Optical rotation measurements were obtained for all amino acid derivatives (Table 2.1).

Compound	Solvent	Concentration (1 = 10 mg/ml)	α_D
5	MeCN	1.00	- 0.47
13	MeCN	0.62	- 1.2
18	MeCN	0.31	+ 5.6
19	$CDCl_3$	0.24	+ 134
22	$CDCl_3$	1.24	+ 19.7
23	MeCN	0.84	- 54.0

Table 2.1

Although **5**, **13** and **18** exhibited α_D values with low moduli, as has been stated this by no means suggested that the material had racemized, furthermore the higher moduli of the other compounds were not indicative of preservation of stereochemistry. Similarly, in the case of CM products, α_D values give no clue as to the diastereotopic weighting of the product mixture and if it is even a mixture at all. Interestingly establishing α_D values for **20**, **21** and **24** proved difficult due to the presence of a red impurity presumably resulting from the ruthenium. In a latter case, no value could be obtained due to this impurity effectively blocking out light at the path length used. Given the fact the sole use of α_D values is to provide a reference value, it was judged irresponsible to report them. A number of protocols for the removal of these highly coloured by-products have been published,[106-108] as has the report of a PEG-conjugated catalyst,[109] whose by-products are simply removed by aqueous workup, and a polymer immobilized catalyst.[110] These procedures were investigated, however were found to be of limited use in the purification of our CM products.

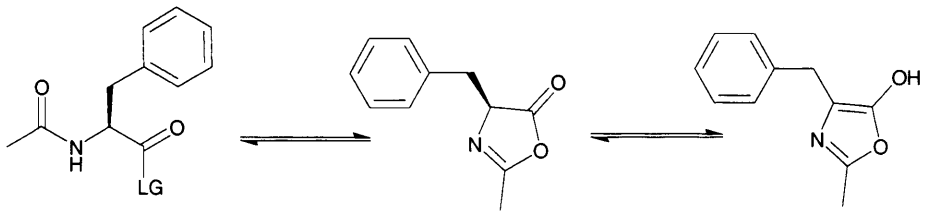
Given the fact that the CM of single amino acid derivatives is a model study, it was decided that these impurities could reasonably be ignored, especially as the proton and carbon NMR spectra were of good quality and exhibited no spurious peaks. It was also envisioned that when applied to large peptides there would be a lower concentration of

ruthenium by-products by weight, as the molecular weight of the peptides involved is much greater.

2.3.2 – Analysis of Stereochemistry of CM Product ***20***

As the CM products have two chiral centres, racemization at either centre will result in diastereoisomers, which will differ in physical properties and thus be detected by NMR analysis. CM product **20** appeared to be a single diastereoisomer by TLC, and there was no evidence of diastereoisomers in the ^{1}H or ^{13}C NMR spectra when the analysis was performed in d_6-DMSO. DMSO is viscous relative to many other common NMR solvents and this often results in peak broadening. In light of this NMR spectra were obtained in d_4-MeOH at 500 MHz, and under these conditions diastereoisomers were very clearly visible.

It was necessary to ascertain where racemization had occurred. To this end, *R*-**18** was prepared in the same manner as **18** using Ac-D-phenylalanine. Firstly, a mixture of the two independently synthesized enantiomers was examined by chiral HLPC in order to determine the chiral separation. The individual compounds were analysed, however were shown to have undergone full racemization. In both cases both enantiomers were present in approximately equal amounts, determined by UV. Racemization occurred presumably *via* the oxazolone rearrangement discussed in the introduction (Scheme 2.22).



Scheme 2.22 – Racemization of activated acetyl phenylalanine *via* the oxazolone

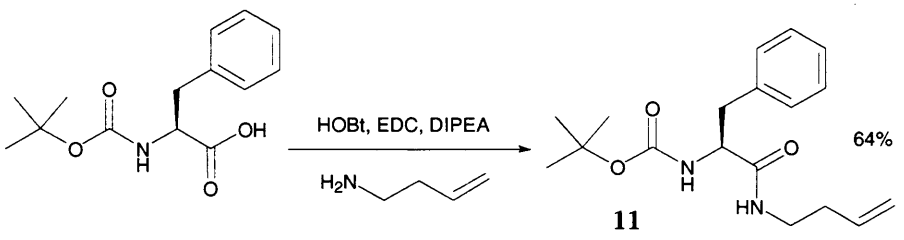
Given that **18** had been shown to have racemized prior to the metathesis step, the effect metathesis has on this centre cannot be determined. In order to determine the likelihood of racemization induced by the CM conditions, access to the non-racemic amino acid derivatives was required.

2.3.3 – Model Study Using a Boc-Protected Amino Acid

Boc-protected amino-acids were chosen for investigation as they are less susceptible to racemization than acetyl-protected derivatives. Indeed this factor, along with their facile deprotection, results in their widespread use in peptide-synthesis. Furthermore, given the frequent occurrence of Boc-protected amines in peptide chemistry we were keen to investigate their compatibility with metathesis.

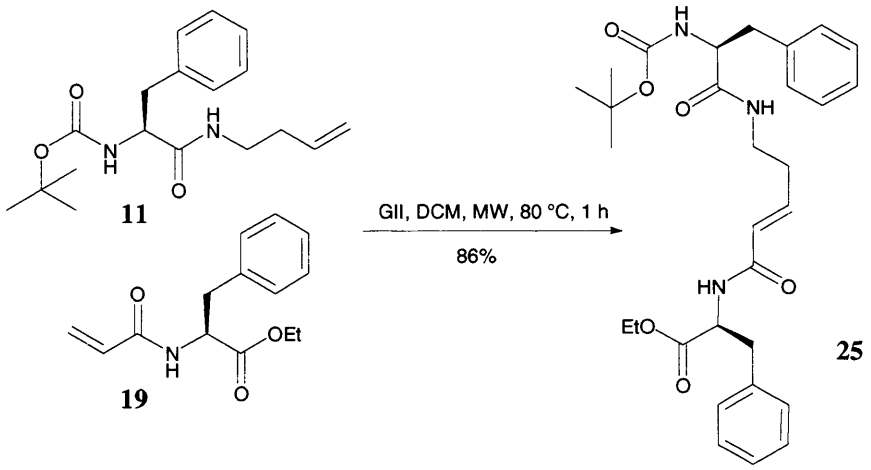
Their greater stability to racemization results from two factors, firstly the steric bulk of the ^{t}Bu group, and secondly the electronics of the carbamate. The steric bulk of the ^{t}Bu group makes the oxazolone formation unfavourable. The overlap between the lone pairs of the carbamate oxygen and the C=O π^* orbital is not significant due to difference in energy. Therefore the oxygen overall has a more inductive effect, polarising the σ bond, and reducing the electron density around the carbonyl carbon atom. This in turn results in a more even distribution of π electrons in the carbonyl and therefore, despite the appropriate energy of the nitrogen lone pair for interaction with the C=O π^* orbitals of the acetyl protected amine, it has a far less favourable overlap in the carbamate due to the higher energy of its C=O π^* orbital. π-Electron density has a much smaller coefficient on the oxygen in the carbamate. Therefore carbamates are far less prone to racemization *via* the oxazolone than amides.

Boc-protected phenylalanine was chosen for investigation, as its synthesis had been reported,[93] and was *C*-functionalized in the same manner as its acetyl-protected counterpart, yielding **11** in 64% (Scheme 2.23).



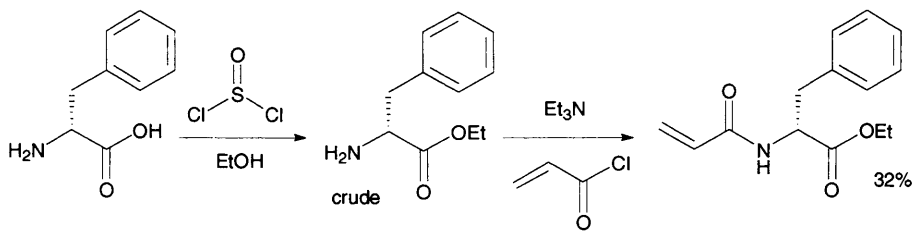
Scheme 2.23 – Synthesis of **11**

CM between compounds **11** and **19** was chosen for this model study. The reaction was carried out using microwave irradiation (Scheme 2.24). The reaction time was greatly reduced, and a full discussion of the employment of microwave irradiation will be given in the following section (2.5). From this point on, CM reactions using convection heating will be referred to as such, likewise microwave enhanced reactions, to avoid ambiguity.



Scheme 2.24 – Microwave-enhanced CM

To evaluate the stereochemical outcome of the CM process, it was necessary to obtain all four diastereoisomers of **25**. The appropriate functionalized amino acids were prepared from the enantiomerically pure Boc amino-acids and amino-acid esters as described previously. Enantiomerically pure Boc *R*-and *S*-phenylalanine and *S*-phenylalanine ethyl ester were obtained from commercial sources, whereas the *R*-phenylalanine ethyl ester derivative, ***R*-19** was synthesized in 32% yield by treatment of *R*-phenylalanine with thionyl chloride in ethanol, and subsequent subjection of this crude product to acryloylation conditions as previously described (Scheme 2.25).



Scheme 2.25 – Synthesis of *R*-**19**

The remaining diastereoisomers were synthesized by CM. Consistent with there being no racemization in the synthesis of the cross-metathesis product, enantiomers exhibited identical carbon NMR spectra, (*RR* identical to *SS*; *SR* identical to *RS*) whereas those of the diastereoisomers differed markedly. Figure 2.9 shows the four diastereoisomers, and typical ^{13}C NMR shift differences (full ^{13}C data given in Chapter 4).

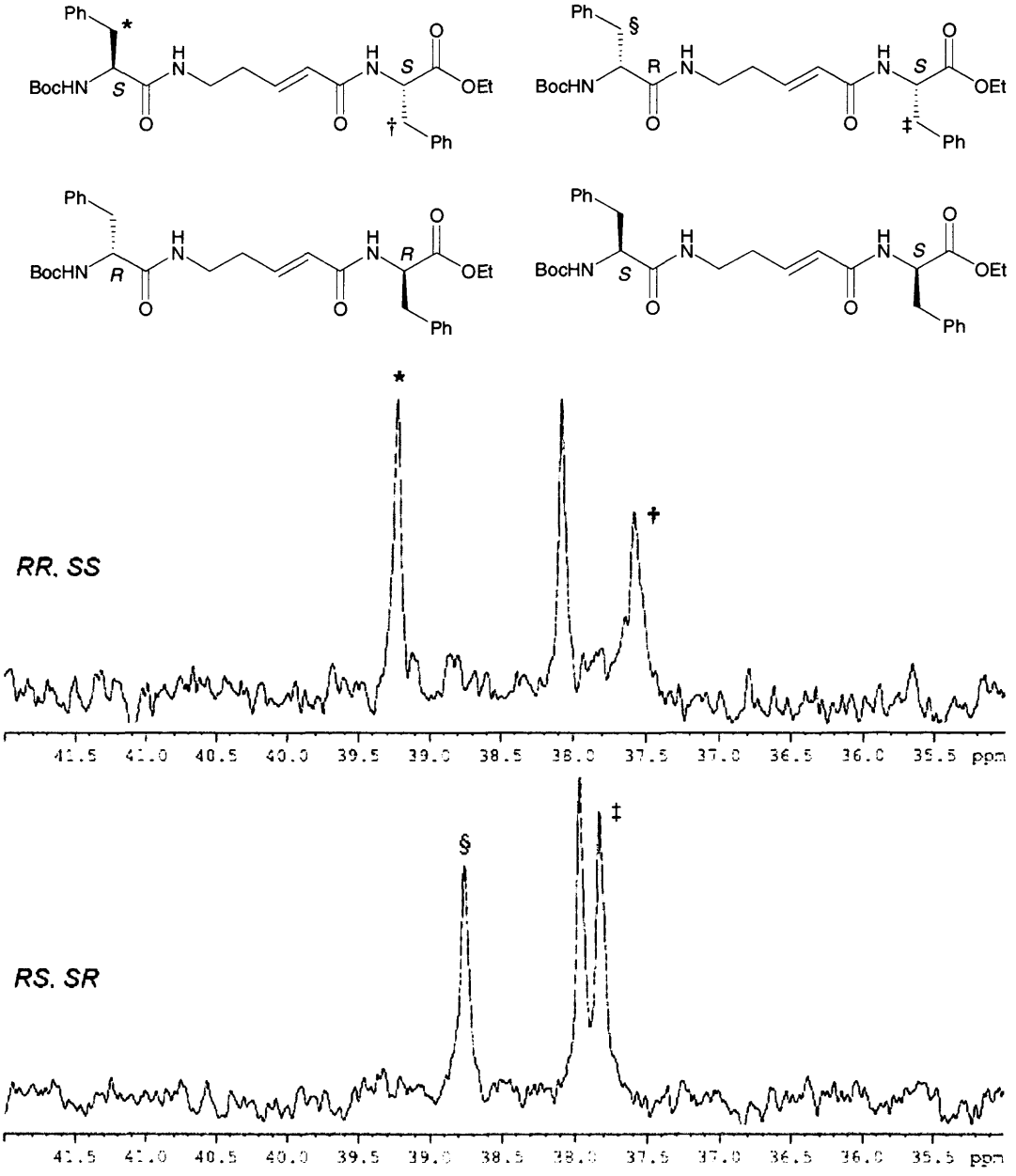


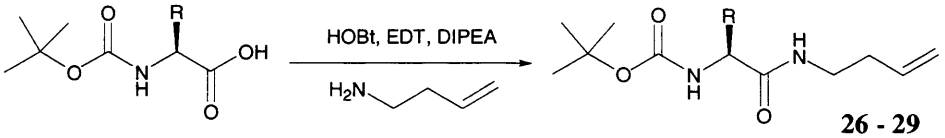
Figure 2.9 – Diastereoisomers of **27** and ^{13}C NMR spectra

It was thus confirmed that stereochemistry is conserved in this case and it is assumed that the stereochemical integrity is maintained in all cases.

2.4 – Extension of Methodology using Convection Heating

2.4.1 – Synthesis of Boc Amino-Acid Derivatives

At this point there were two problems to be addressed. Firstly, synthesis of *N*-acryloyl amino acid esters had proved problematic. Secondly, the *N*-acetyl phenylalanine derivative had been shown to have racemized. However the latter problem could be overcome by utilisation of the analogous Boc protected derivatives, and so it was decided to generate several Boc-protected *C*-terminal homoallyl amide substrates. Four compounds were synthesized according to Scheme 2.26 (Table 2.2).



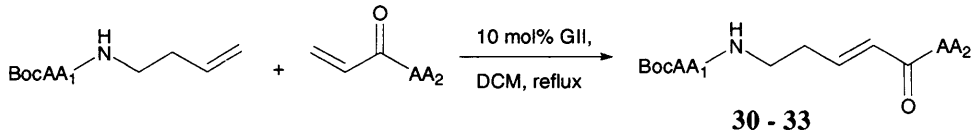
Scheme 2.26 – Synthesis of *C*-terminal homoallyl amide species **26-29**

Compound	Amino Acid	Yield/%
26	BocHis(Tos)	73
27	BocTyr(Bzl)	61
28	BocArg(diZ)	54
29	BocSer(Bzl)	46

Table 2.2

2.4.2 – Scope of CM Ligation

A variety of CM reactions were now carried out, using a protocol involving refluxing DCM and 10 mol% **GII** (Scheme 2.27). It is fairly common for metathesis reactions to be carried out with relatively high catalyst loadings. In all cases a small excess of *N*-acryloyl amino acid was used, and this was monitored by TLC. The results are shown in Table 2.3.



30 - 33

Scheme 2.27 – General CM reaction

Compound	AA_1	AA_2	Yield
30	His(Tos)	PheOEt	16
31	Arg(di-Z)	PheOEt	70
32	Tyr(Bzl)	PheOEt	67
33	Ser(Bzl)	ProOMe	49

Table 2.3

NMR spectra of these products were consistent with a trans alkene (J_{trans} = 15 Hz).

31 and **32** were obtained in acceptable yield of 70% and 67% respectively. **30** and **33**, on the other hand, were obtained in disappointing yields of 16% and 49% respectively which was deemed unacceptable as it falls within the realm of statistical CM reactions.

The synthesis of **30** was curious as both starting materials were still visible by TLC after 48 h. This was in contrast to the expectation that the more reactive olefin would homodimerize rapidly. Indeed in analogous reactions we observed complete consumption of **31**, **32** and **33** after a few hours, which is assumed to be *via* homodimerization. In the present case one possibility is that the catalyst is being poisoned or its activity significantly compromised, possibly by the tosyl-protected histidine moiety.

As both starting materials were present after 48 h, the reaction was terminated, and upon purification the CM product **32** was obtained in 16% yield. In order to determine whether the reaction was slow, due to the specific side-chain functionality, the reaction was carried out at reflux for 6 days. The isolated yield improved to 29% and further improvement to 36% was observed with an increase in catalyst loading (16 mol%) and a change to dichloromethane as solvent. It is hard to ascertain the nature of this apparent

deactivation process. It is possible that the His(Tos) side chain permanently deactivates the catalyst or competitively binds to ruthenium. It is interesting to note, however, that there is a literature report which describes the lack of reactivity of **26** with **GI**.[93] Further investigation was judged unnecessary as this phenomenon was not observed in any other examples; on no occasion was any other reactive olefin isolated.

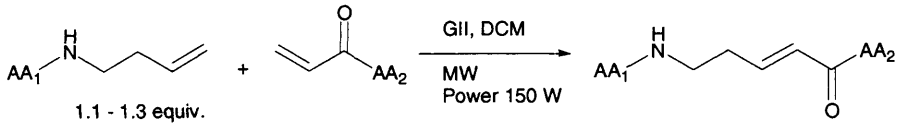
2.5 – Microwave Enhancement and Optimization

2.5.1 – Microwave-Enhanced Cross-Metathesis

Microwaves has been widely used in modern synthetic chemistry and in many cases has been reported to produce dramatic increases in the rate of reactions.[111] We noted that it has been reported to greatly increase the rate of both self cross-metathesis,[112] and selective cross-metathesis.[113]

The effectiveness of a solvent in converting microwaves to heat can be measured by considering the ratio of the amount of input microwave energy that is lost to the sample as dissipated heat to the molecules ability to store electric charge.[114] Thus polar solvents such as dimethylformamide (DMF) and water heat rapidly under microwave conditions whereas solvents such as DCM interact very little with microwaves. The idea of a 'microwave effect' is hotly contested,[115] and attempts have been made to rationalize unusual experimental observations.[116, 117] It is possible that the rapidity of the reaction simply results from a combination of increased pressure and heat dissipated to the system by the irradiated solvent. Indeed, in the field of microwave-promoted metathesis, studies have both supported[118] and contradicted[119] this idea.

However irrespective of the detailed nature of the observed affects we decided to embark upon a study to determine the impact of microwave radiation on our metathesis protocol. Various conditions were attempted across a range of substrates in an effort to determine the effect of microwave irradiation and thus establish a good starting point for optimization (Scheme 2.28). Results are given in Table 2.4.



Scheme 2.28 – Initial conditions for microwave-enhanced CM

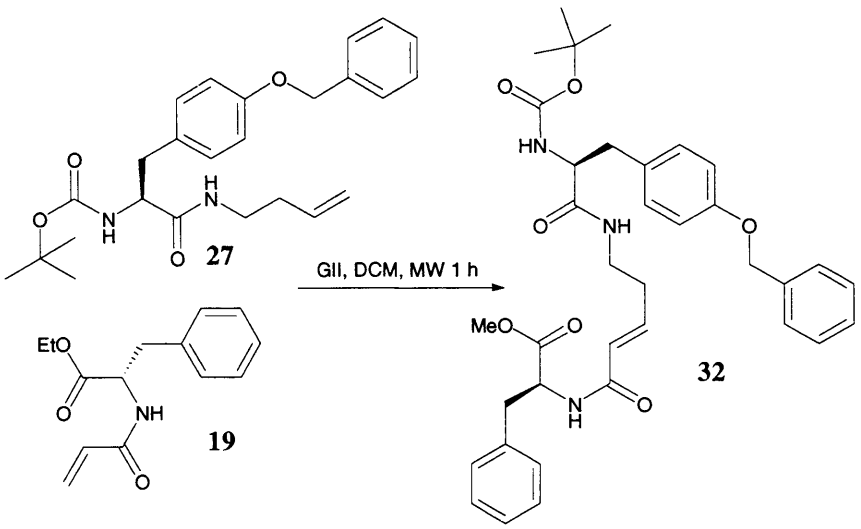
Compound	AA_1	AA_2	T/°C	Time	Mol% **GII**	Yield/%
25	BocPhe	PheOEt	80	1 h	19	86
24	AcPhe	ValOEt	90	2 x 15 min	9	55
20	AcPhe	PheOEt	90	1 h	19	71
30	BocHis(Tos)	PheOEt	90	2 x 15 min	19	41
32	BocTyr(Bzl)	PheOEt	90	4 x 15 min	19	57
31	BocArg(diZ)	PheOEt	90	1 h	17	43
33	BocSer(Bzl)	ProOMe	60	2 h	11	35

Table 2.4

From these results, it appears that in most examples the yield has been diminished upon use of microwave conditions. It does, predictably, increase reaction rates significantly in all cases, and this alone was enough to prompt us to investigate the use of microwave irradiation further.

2.5.2 - Optimization

It was clear that a specific optimization study was required. The synthesis of **32** (Scheme 2.29) was chosen for this as the yield under microwave irradiation was 10%, which gave plenty of opportunity for optimization. Results are shown in Table 2.5.



Scheme 2.29 – Optimized CM reaction

	Mol(**27**)/ Mol(**19**)	Mol% **GII**	Temp (°C)	Power (W)	Yield (%)
i	1.3	19	90	150	57
ii	1.3	18	80	150	55
iii	1.3	18	90	300	66
iv	0.83	26	90	300	61
v	1.3	18[a]	90	300	36
vi	2.1	20	90	300	52
vii	0.83	18[b]	90	300	58
viii	1.3	18	100	300	82
ix	1.3	18	110	300	59
x	1.3	18	100	*n/a*[c]	58
xi	1.3	18	95	*n/a*[c]	77

[a] **HG** used. [b] Catalyst added in 2 x 9 μmol portions. [c] Used a different microwave unit without adjustable power.

Table 2.5

CM reactions were carried out for 30 minutes then degassed by bubbling argon through the reaction mixture for 30 seconds before further irradiation for 30 minutes. Degassing was employed to remove the ethene, which would otherwise remain in the sealed microwave vessel. For entry (ii), the experiment was monitored at 30 minute intervals

and TLC analysis indicated that **19** persisted in the reaction mixture. From this study we decided on a protocol involving two periods of 30 minutes irradiation interjected with a brief period (30 seconds) of 'degassing' with argon.

This study showed that microwave power and the stoichiometric ratio of olefins had an effect on the yields. An increase in yield was obtained by increasing the power to 300 W, this was apparent from comparison of the results from entries (i) and (iii). The power should merely affect the rate at which the solvent attains the desired temperature, and therefore this effect on yield was unexpected. Given this observed increase in yield, however, 300 W was chosen for all subsequent microwave reactions. It was concluded that 1.3 equivalent excess of **19** gave generally better yields, an observation that was pleasing from the point of view of application to peptides.

Entry (v) showed that the use of **HG** in place of **GII** results in a considerably lower yield compared to the other examples. Hence we elected to utilise **GII** in all further experiments.

The variation of temperature had the greatest effect on the outcome of the reaction. Initially, temperatures above 90°C were avoided as *t*-butyl carbamates are prone to thermal degradation.[120] However when the temperature was increased to 100°C a significant increase in yield was obtained, (compare entries (iii, 66%) and (viii, 82%)). This suggests that one of the key steps in the reaction mechanism of this particular metathesis has a high energy-barrier. Indicative of the threshold of the compounds' thermal stabilities is entry (ix), which shows a significant drop in yield to 59% resulted from a 10°C increase to 110°C.

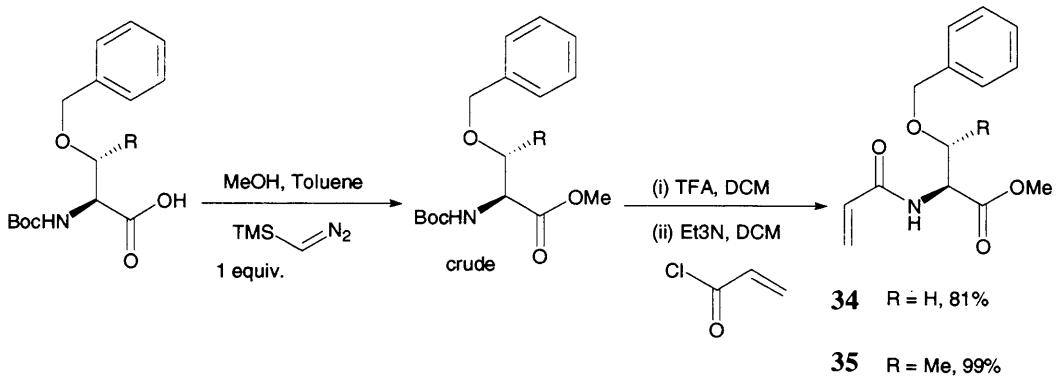
A satisfactory optimization had therefore been completed. The high-yielding example was repeated, however at the time the usual microwave unit was unavailable. Disappointingly, the use of a different microwave unit (Biotage Initiator 60) yielded 58%, entry (x). It was believed that this difference may reflect the inferior temperature control and in the early phases of the reaction a higher temperature may be operating. In this new

apparatus, another attempt was made this time utilising 95°C and, gratifyingly, 77% CM product was obtained. Whilst the results were not directly comparable, this demonstrated that under optimized conditions acceptable yields could be attained.

2.6 – Application of Optimized Conditions

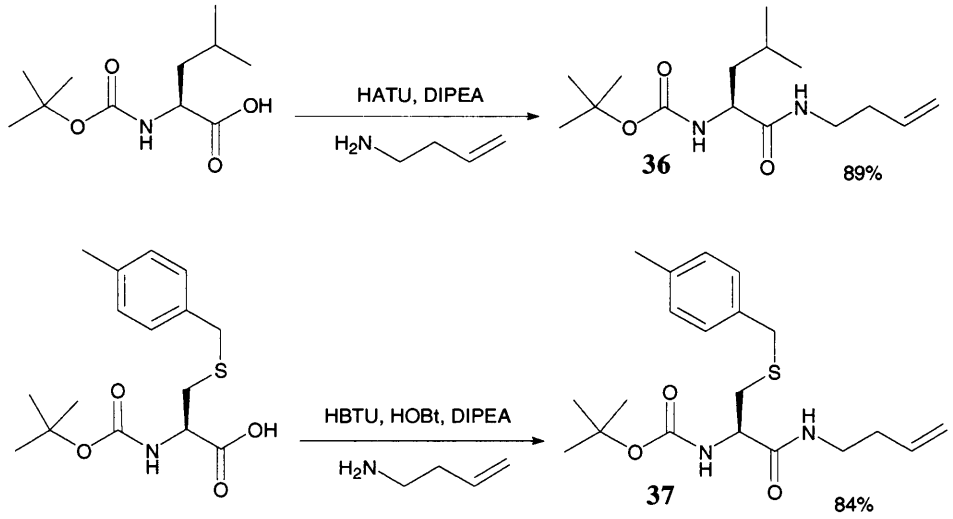
2.6.1 – Preparation of Additional Substrates

Prior to the application of the optimized conditions to all substrates, some additional compounds were prepared. *N*-Acryloyl- serine and threonine derivatives **34** and **35** were synthesized from the Boc-protected amino acids according to Scheme 2.30.



Scheme 2.30 – Synthesis of **34** and **35**

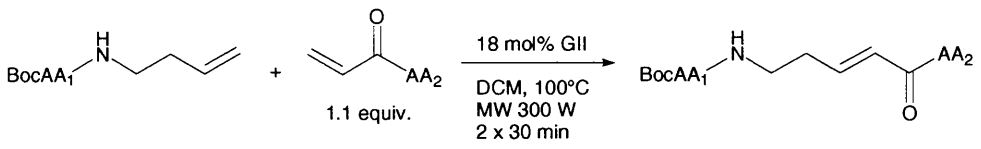
Additionally, Boc leucine and Boc S-4-methylbenzyl cysteine were *C*-functionalized according to the reaction conditions described in Scheme 2.31, in yields of 89% and 84%, respectively.



Scheme 2.31 – Synthesis of **36** and **37**

2.6.2 – Application of Optimized Conditions

The optimized conditions were applied to the full range of substrates with one modification. We opted to use 1.1 equivalents as opposed to 1.3 of the *N*-acryloyl amino-acid esters in most cases, as we wanted to the protocol to be as economical as possible for application to large peptides. Additionally, as an excess of 1.3 had given good results in the optimization studies, it was assumed that 1.1 equivalents would be similarly successful. The results for the CM reactions (Scheme 2.32) are shown in Table 2.6.



Scheme 2.32 – Optimized CM conditions

	Compound	AA_1	AA_2	Yield/%
i	***SS*-25**	*S*-Phe	*S*-PheOEt	63
ii	**38**	Phe	ValOEt	34
iii	**38**	Phe	ValOEt	30[a]
iv	**31**	Arg(di-Z)	PheOEt	43
v	**31**	Arg(di-Z)	PheOEt	40
vi	**31**	Arg(di-Z)	PheOEt	55[b]
vii	**33**	Ser(Bzl)	ProOMe	50
viii	**39**	Ser(Bzl)	PheOEt	73
ix	**40**	Leu	Thr(Bzl)OMe	61[a]
x	**41**	Leu	Ser(Bzl)OMe	75[a]
xi	**42**	Cys(4-MeBzl)	PheOEt	51

[a] Used a different microwave unit without adjustable power. [b] 1.5 equivalent excess of *N*-acryloyl amino-acid used

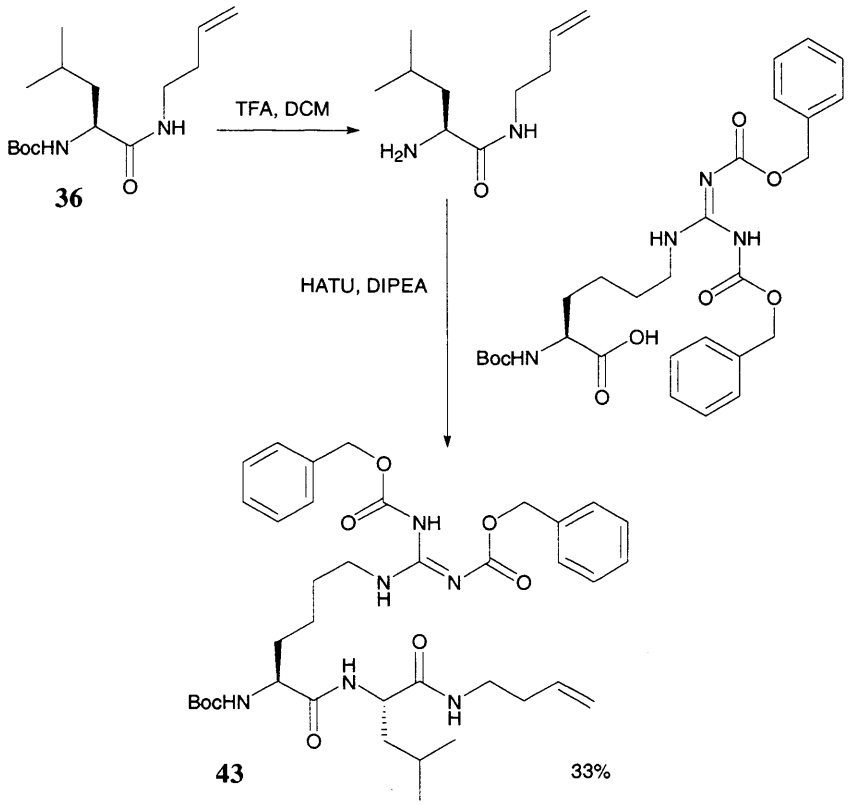
Table 2.6

It is apparent from these results that individual optimization may be necessary in order to maximise individual yields. However for the purposes of this study this was deemed unnecessary and we decided to focus attention on exploration of the wider scope of the methodology. It should be noted that **41** existed as two diastereoisomers (approximately 15:3, by ^{13}C NMR), visible by NMR. This is presumably due to partial racemization of serine during coupling to homoallyl amine *via* the oxazolone. The high propensity of serine to racemize has been reported.[121]

This model study has demonstrated the wide applicability of the technique in generating peptidomimetic structures of equivalent length to four amino acids from functionalized single amino acids. It must be highlighted that such structure could potentially be incorporated into peptide sequences using existing SPPS protocols, much the same as Kelly's peptidomimetic.[98]

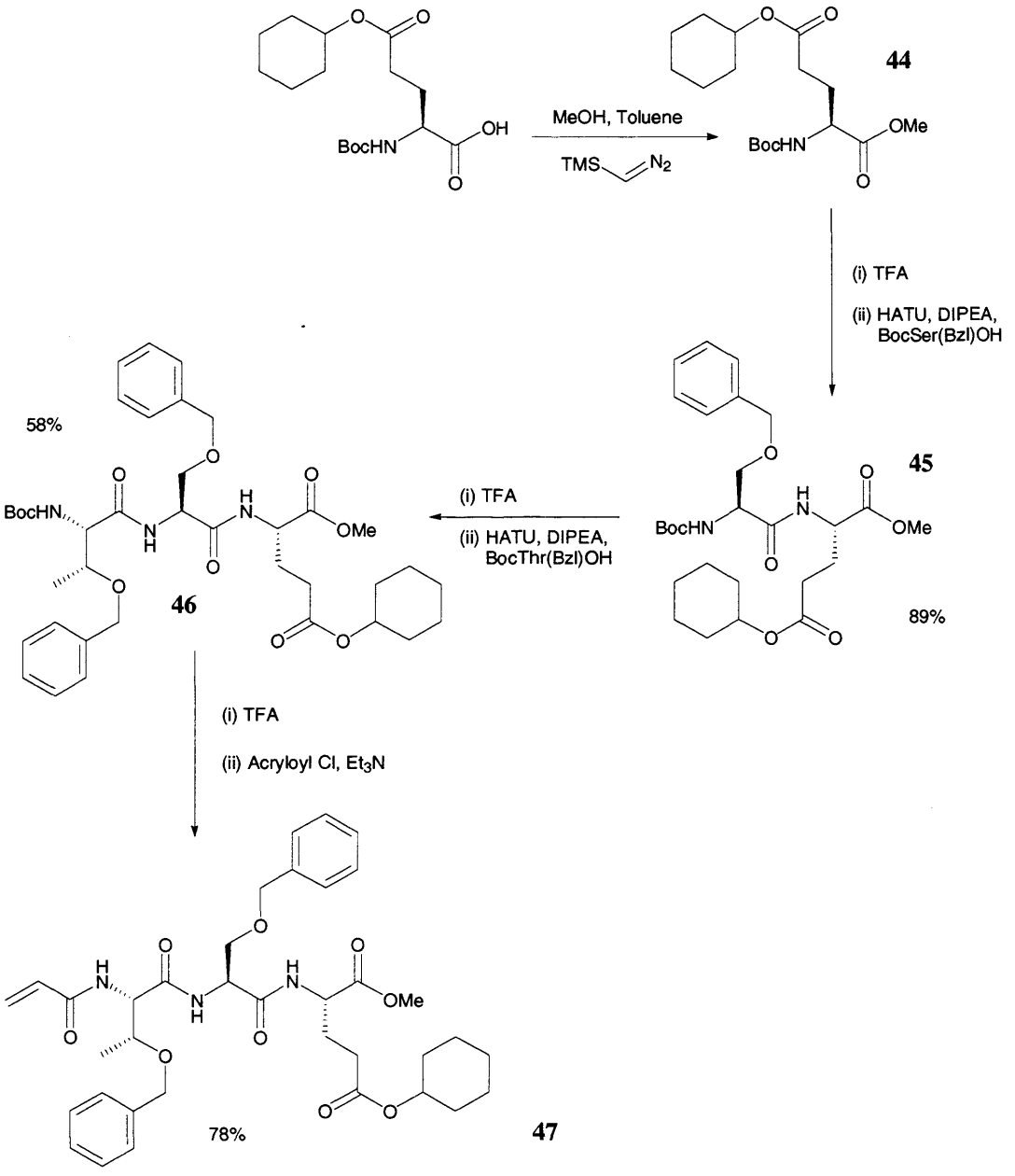
2.7 – Application to a Larger Peptide

As encouraging as these results were, single amino acids are not sufficiently representative of larger peptides for us to infer applicability to large peptides or proteins. Therefore, before attempting the synthesis of a protein using this novel ligation, it was decided to attempt the ligation with larger peptides. Thus olefin-derivatized dipeptide **43** was synthesized from **36** using solution-phase procedures, according to Scheme 2.33. 33% of pure material was obtained.



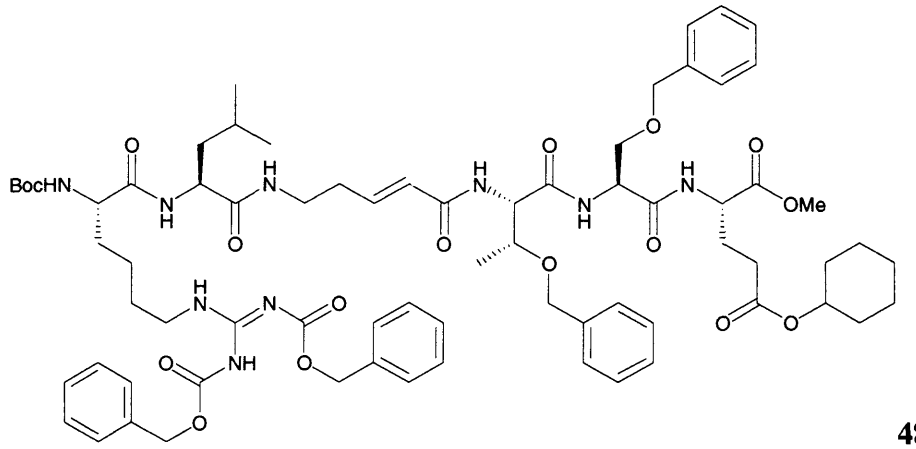
Scheme 2.33 – Synthesis of functionalized dipeptide **43**

The choice of amino acids was not arbitrary – they correspond to those found on the *N*-terminal side of the linkage site proposed in the retrosynthesis analysis of Crambin. The choice of linkage site is discussed in Chapter 3. An appropriately functionalized tripeptide corresponding to the residues found on the *C*-terminal side of Crambin was also synthesized. This was prepared using a solution-phase Boc-protection approach, according to Scheme 2.34.



Scheme 2.34 – Synthesis of functionalized tripeptide **47**

Compounds **43** and **47** were subjected to the optimized metathesis conditions. LCMS and evaporative light-scattering detection analysis of the crude mixture suggested the CM product **48** (Figure 2.10) had been produced in 67% yield, however only 38% was isolated using flash column chromatography on silica gel.



48

Figure 2.10 – CM product **48**

This result was the first example of CM performed on peptides of this size, and also served as a very encouraging demonstration of the methodology in the presence of a host of functional groups. Interestingly, in this case the product was obtained as an inseparable 1:8 mixture of *cis* and *trans* isomers. It is difficult to say why in this case alone geometric isomers were obtained; perhaps some secondary structural effects decrease the relative free energy of the *cis* configuration.

2.8 – Conclusion

From these studies,[122] it can be concluded that this cross metathesis protocol is effective in the presence of a variety of amino acids and can be assumed to result in no appreciable racemization. It does appear, however, to require optimization in each case, and some specific amino acids give very poor CM yields, for example with valine, proline and tosyl-protected histidine. As for application of this methodology, it can be viewed, variously, as a new ligation technique, a method of inserting a peptidomimetic which lacks a main chain amide bond, similar to the work of Kelly, and a way by which a site capable of further functionalization and manipulation may be incorporated into a peptide. The employment of this methodology will involve several considerations, chiefly the effect that replacement of two amino acids with a peptidomimetic will have on protein structure and function. Furthermore, the peptide fragments must be synthetically accessible, a factor which may influence the division of the protein. We shall now turn to the application of this new methodology in the synthesis of the plant protein, Crambin.

Chapter 3 – Towards the Total Synthesis of Crambin

3.1 – Introduction

The Plant protein Crambin was an ideal candidate for application of the metathesis ligation described in Chapter 2. This is because it is small, and therefore the segments should be reasonably accessible by SPPS. Indeed such syntheses have been reported.[88, 89] Moreover, in terms of structure and folding, Crambin is one of the most studied of all proteins (See section 1.6).

3.1.1 – Choice of Ligation Site

The choice of ligation site in the application of metathesis ligation to the chemical synthesis of a protein requires the consideration of two factors. These are availability of fragments and the likely effect that insertion of the peptidomimetic linker would have on the structure. The latter is hard to predict, however substitution in regions of defined secondary structure is likely to cause severe perturbations resulting from loss of an amide bond and thus hydrogen bonding ability. The peptidomimetic generated upon ligation lacks analogous side-chains, and thus most closely resembles two glycine residues (Figure 3.1). Any Gly-Gly unit has potential for ligation.

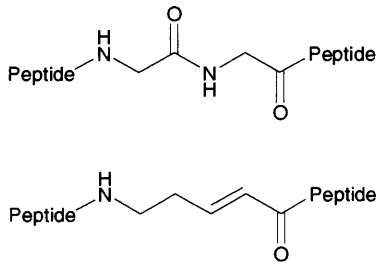


Figure 3.1 – Gly-Gly and resultant linker, for comparison

In most cases a Gly-Gly unit at an appropriate site will not be available, and so in each case the structural involvement of side chains in the native protein will have to be examined. In the long term it is not unreasonable for any 'retrosynthetic analysis' of a protein to require this type of strategic planning.

Crambin has a size accessible by traditional SPPS, and so the question of fragment size did not have a bearing on the choice of ligation site. Ideally, however, we wanted to divide the protein approximately in half, so that the two different methods of peptide functionalization, *C*-terminal and *N*-terminal, could be attempted on reasonably sized peptides. There are no adjacent glycine residues in the amino-acid sequence of Crambin, and so it was necessary to consider replacement of different residues.

Even if the ligation site is located outside a region of secondary structure, the replacement of two amino acids with the peptidomimetic has the potential to interfere with non-covalent interactions in the protein's tertiary structure. Therefore, it was necessary to consider specific examples and make a qualitative prediction of the likely impact of our proposed 'synthetic mutation'.

Figure 3.2 shows a cartoon of the tertiary structure of PL-form Crambin, based on the high-resolution crystal-structure at 150 K.[75] Two possible ligation sites were considered – both falling outside well-defined secondary-structure, and dividing the protein into suitably large segments. These are shown in yellow.

Figure 3.2 – Cartoon of PL-from Crambin

The first is located in the region between an α-helix and a β-sheet, replacing Gly[31] and either Thr[30] or Cys[32]. All cysteine residues form disulfide bridges in folded Crambin, and so replacement of Cys[32] was not considered due to the importance of these moieties in maintaining the structural integrity of the protein. From analysis of the high-resolution crystal-structure it could be seen that both the Thr[30] side-chain and the Gly[31] are involved in hydrogen bonding. Figure 3.3 shows the hydrogen bonds in the region in yellow. The bond lengths are given in Angstroms.

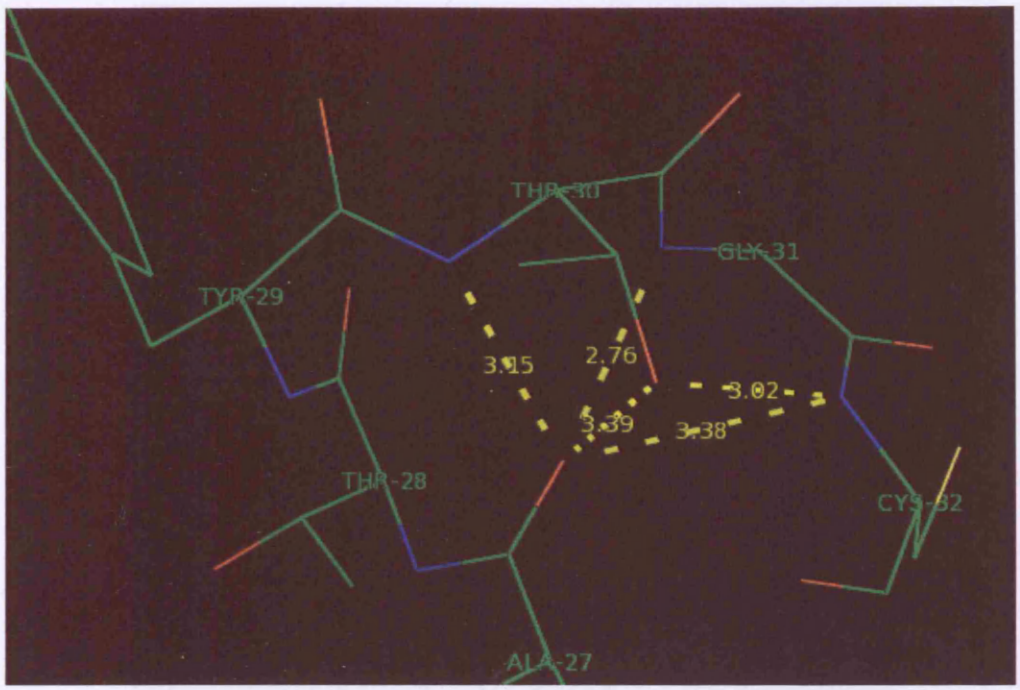


Figure 3.3 – Hydrogen bonds in Cram[27-32] shown in yellow

Although we were attempting to introduce the non-native linker in unstructured regions, it was clear that it would be hard to avoid their disruption altogether – clearly in this region hydrogen bonding is supporting the turn-like structure.

The second potential ligation site occurs between two α-helices, potentially replacing Gly[20] and either Pro[19] or Thr[21]. Once again, hydrogen bonding would be disrupted. In the native peptide Thr[21] side-chain is involved in hydrogen bonding, as is Gly[20] in a β-turn-like structure (Figure 3.4).

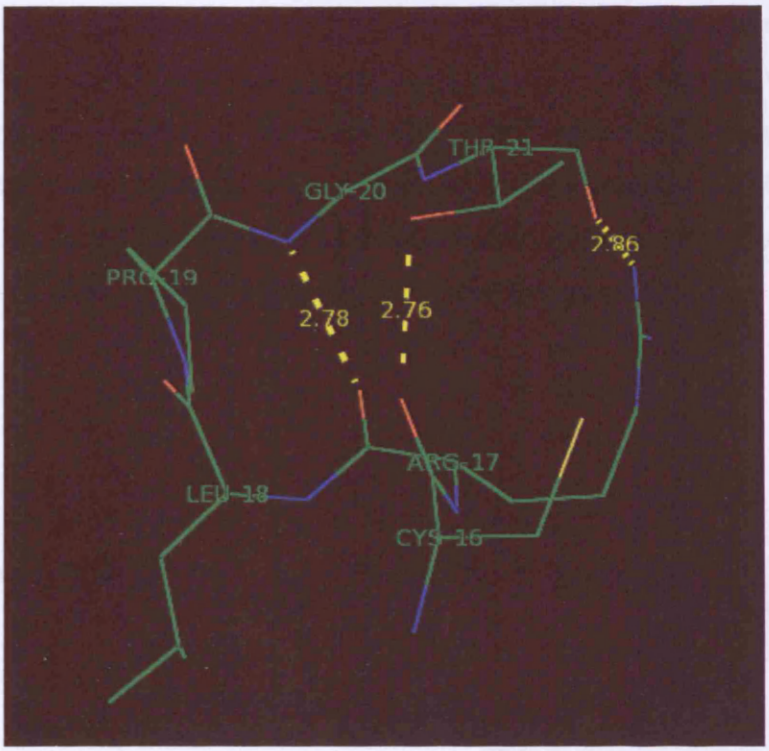


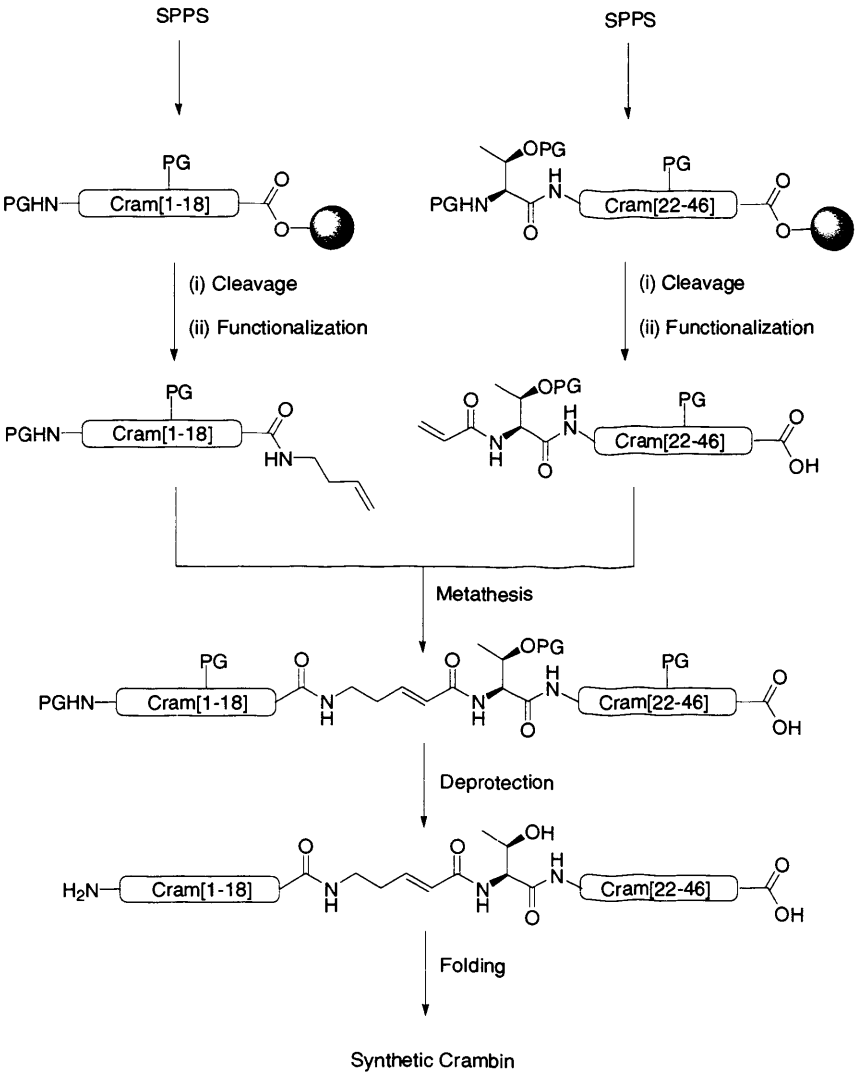
Figure 3.4 – Hydrogen bonds in Cram[16-21]

The Pro[19]-Gly[20] junction was chosen for ligation, as there were fewer hydrogen bonds in this region. It was acknowledged that such a replacement would remove a hydrogen bond in a β-turn-like structure, and that the conformational constraints effected by Pro[19] would be lost. Such a modification, however, could in itself produce interesting results – it would be possible to assess the importance of these features in dictating the overall tertiary structure of Crambin. Indeed as only a single hydrogen bond is removed if the Pro[19]-Gly[20] unit is replaced, it may be possible to attribute any structural perturbations to this alone, making this linkage site the more attractive option.

3.2 Synthetic Strategy

It was reasoned that SPPS should allow easy access to Cram[1-18] and Cram[21-46]. Previous reports of the synthesis of Crambin by NCL utilize a Boc protection, however this relies on the use of hydrofluoric acid for resin cleavage and so the Fmoc protection strategy was chosen for its practicability. Furthermore Fmoc chemistry on chlorotrityl

resins allows for the generation of fully protected peptide fragments *via* cleavage with acetic acid[20-23] (Section 1.2.3). The ligation of peptides *via* CM requires full protection; firstly to prevent catalyst deactivation interactions with thiol and amine functionality, and secondly to render the fragments soluble in DCM. Once synthesized, it was hoped the peptide fragments could be appropriately functionalized. Deprotection could occur after ligation and the synthetic protein subsequently folded (Scheme 3.1).

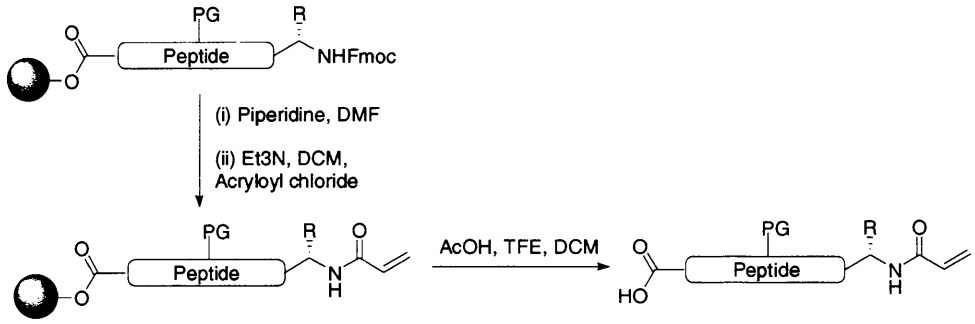


Scheme 3.1 – Proposed route to synthetic Crambin

Despite the fact it is possible to cleave peptide fragments fully protected with subsequent solution-phase functionalization,[20-23] there were far more attractive approaches to

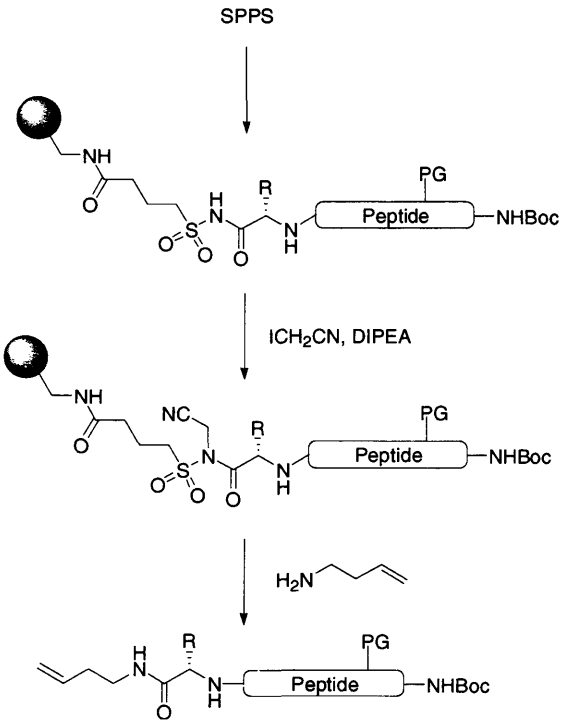
generating the functionalized peptides. These approaches – on-resin acryloylation for *N*-functionalization and a safety-catch linker strategy for *C*-functionalization – avoided solution phase manipulation.

In the case of *N*-functionalization, solution-phase steps could potentially be avoided by direct acryloylation, *i.e.* bypassing Boc protection, cleavage and Boc deprotection. Cleavage with acetic acid, which leaves protecting groups intact, then yields the desired protected peptide (Scheme 3.2).



Scheme 3.2 – On-resin acryloylation of *N*-terminus

Similarly, with the use of a sulfonamide safety-catch linker,[123] *C*-terminal functionalization and cleavage in theory could be achieved in a single step according to Scheme 3.3.



Scheme 3.3 – Simultaneous cleavage and *C*-functionalization

In summary, it was hoped that the appropriately functionalized Crambin segments would be accessible *via* simple SPPS steps and on-resin manipulations. It was decided to investigate the synthesis of both fragments on chlorotrityl resin prior to functionalization in the case of Cram[21-46] or switching to a safety catch approach in the case of Cram[1-18].

3.3 – Synthesis of Fragments

3.3.1 – Introduction

Figure 3.5 shows the proposed ligation site in PL form Crambin. This exhibits Pro[22] as opposed to Ser[22], found in the SI form. Proline residues are favourable in SPPS, indeed 'pseudoprolines'[124] have been shown to facilitate access to difficult sequences by disrupting hydrogen bonding.[125] As the choice was arbitrary, PL form was chosen due to the fact it contains an extra proline residue.

Thr-Thr-Cys-Cys-Pro-Ser-Ile-Val-Ala-Arg-Ser-Asn-Phe-Asn-Val-Cys-Arg-Leu-**Pro-Gly**-Thr-Pro-Glu-Ala-Leu-Cys-Ala-Thr-Tyr-Thr-Gly-Cys-Ile-Ile-Ile-Pro-Gly-Ala-Thr-Cys-Pro-Gly-Asp-Tyr-Ala-Asn

Figure 3.5 – PL form Crambin. The proposed ligation site is shown in bold

All syntheses utilized a Microwave Peptide-Synthesizer (MWPS), which has been shown to be superior to traditional automated peptide synthesizers in terms of minimisation of side reactions, length of coupling times and ability of access difficult sequences.[126] From the outset, methods were set to subject the deprotected amine to two rounds of microwave couplings for proline residues, and any residue being coupled to proline, leucine, isoleucine and valine. This was in anticipation of potentially poor couplings. These could result from steric hindrance caused by a high degree of side-chain branching in the case of leucine, isoleucine and valine, and the conformational inflexibility of proline. Additionally, two rounds of coupling were used in the case of proline residues due to their associated steric issues. In order to examine the peptide purity in each case, a small amount was cleaved from the chlorotrityl resin using TFA:EDT:H_2O:TIS (94:2.5:2.5:1), conditions which simultaneously effect full deprotection. The crude mixtures were precipitated in cold ether, and the washed with cold ether three times. When it was necessary to leave protecting groups intact the milder acetic acid cleavage was used (AcOH:TFE:DCM, 1:1:8).

3.3.2 – Linear Syntheses

3.3.2.1 – Cram[21-46]

The synthesis of Cram[21-46] was attempted. HPLC analysis of the TFA-cleaved material showed it to be impure (Figure 3.6) and no detectable mass peak could be seen in the ES+ mass spectrum.

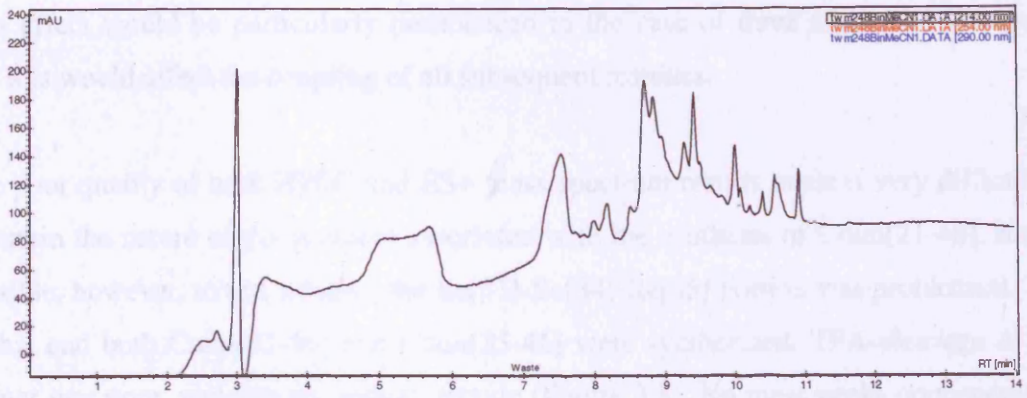


Figure 3.6 – Chromatogram of crude Cram[21-46]
Sequence: TPEALCATYTGCIIIPGATCPGDYAN

The sequence was attempted two further times, firstly by a simple repetition, secondly by modifying the coupling protocols to double the coupling of each residue with the aim of improving the purity of the product. Unfortunately, with double coupling of each residue, the chromatogram still exhibited several peaks (Figure 3.7), and no detectable mass peak could be seen in the ES+ mass spectrum.

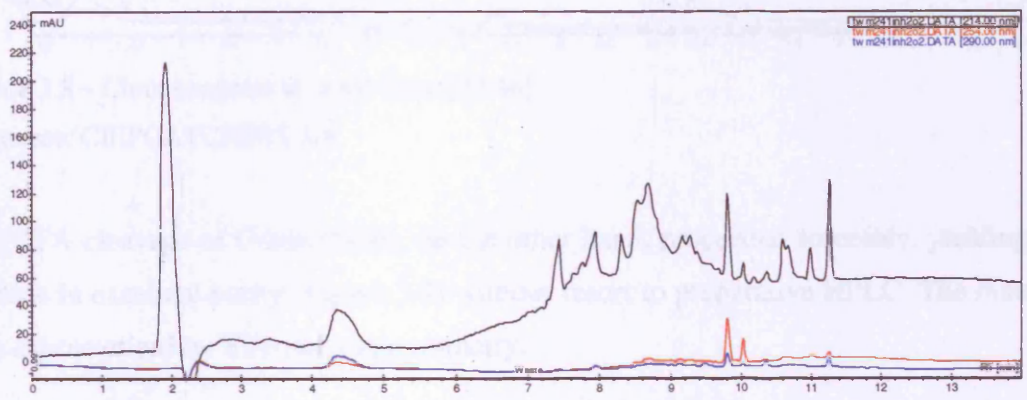


Figure 3.7 – Chromatogram of crude Cram[21-46]
Sequence: TPEALCATYTGCIIIPGATCPGDYAN

It appeared that the undesirable product was not a simple deletion sequence, and that there were multiple coupling issues. The Ile[33]-Ile[34]-Ile[35] segment was considered to be a potential cause of the problems. Hydrophobic residues tend to associate through hydrophobic interactions, and this can render the amino terminus inaccessible to coupling.

This effect would be particularly pronounced in the case of three adjacent isoleucines, and this would affect the coupling of all subsequent residues.

The poor quality of both HPLC and ES+ mass spectrum results made it very difficult to ascertain the nature of the problem associated with the synthesis of Cram[21-46]. It was possible, however, to test whether the Ile[33]-Ile[34]-Ile[35] portion was problematic and to this end both Cram[32-46] and Cram[35-46] were synthesized. TFA-cleavage of the former was poor, yielding an impure peptide (Figure 3.8). No mass peaks corresponding to Cram[32-46] were visible in the ES+ mass spectrum.

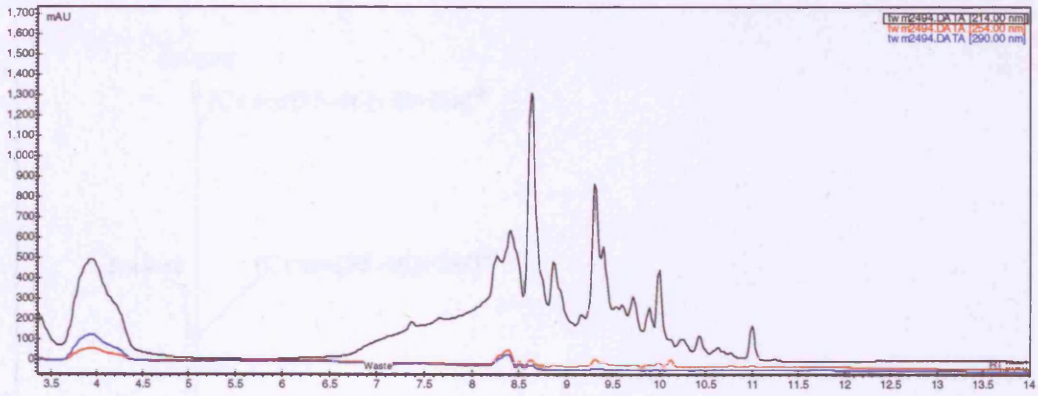


Figure 3.8 – Chromatogram of crude Cram[32-46]
Sequence: CIIIPGATCPGDYAN

The TFA-cleavage of Cram[35-46], on the other hand, proceeded smoothly, yielding the peptide in excellent purity (Figure 3.9) without resort to preparative HPLC. The material was characterised by ES+ mass spectrometry.

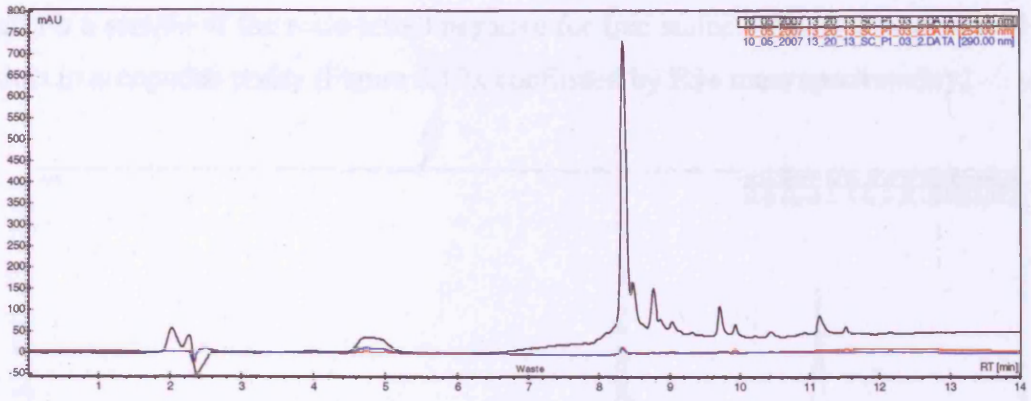


Figure 3.9a – Chromatogram of crude Cram[35-46]

Sequence: IPGATCPGDYAN

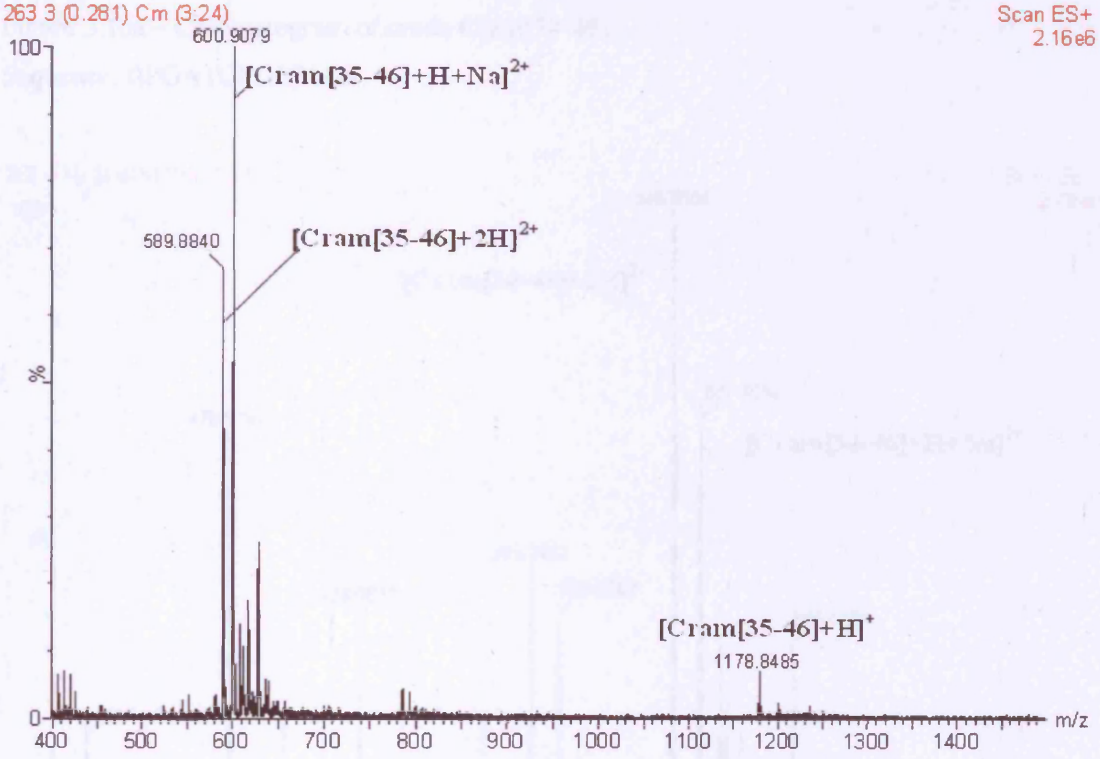


Figure 3.9b – MS spectrum of crude Cram[35-46]

Calculated m/e for $[(IPGATCPGDYAN)+H]^+$: 1178.515

This gave good evidence that the difficulties were associated with isoleucine couplings. Coupling of the next isoleucine was carried out manually, without microwave irradiation. The completeness of coupling was being assessed by Kaiser test[127] (Section 4.2.2.3).

After 3 h a sample of the resin tested negative for free amine. TFA-cleavage yielded the product in acceptable purity (Figure 3.10), confirmed by ES+ mass spectrometry.

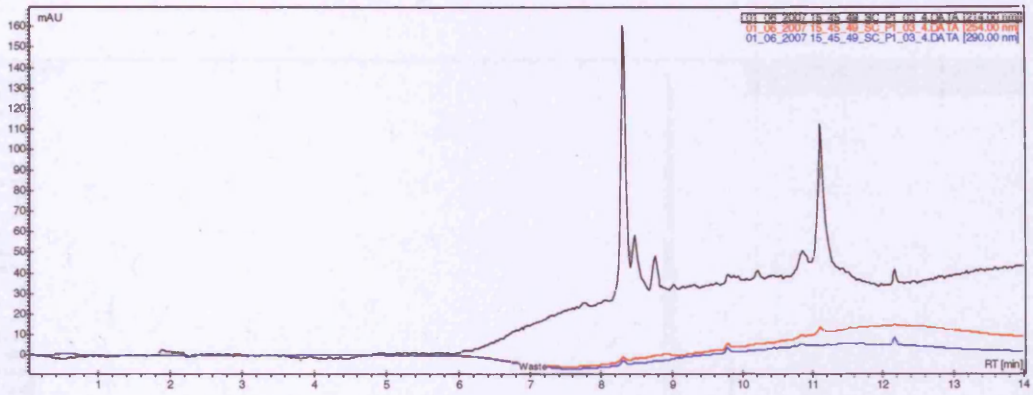


Figure 3.10a – Chromatogram of crude Cram[34-46]

Sequence: IIPGATCPGDYAN

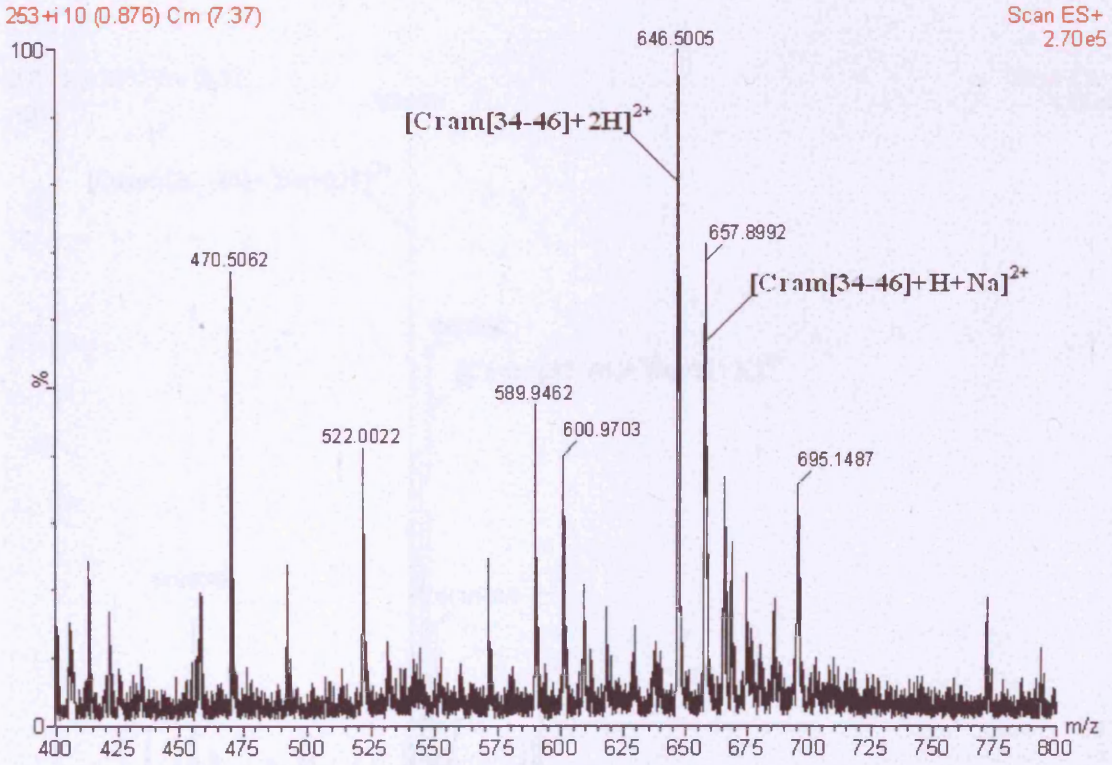


Figure 3.10b – MS Spectrum of crude Cram[34-46]

Calculated m/e for $[(IIPGATCPGDYAN)+2H]^{2+}$: 646.304

The coupling of Ile[33] and Cys[32] were carried out manually in a similar manner to the coupling of Ile[34]. TFA-cleavage afforded Cram[32-46] in excellent purity, confirmed by ES+ mass spectrometry (Figure 3.11).

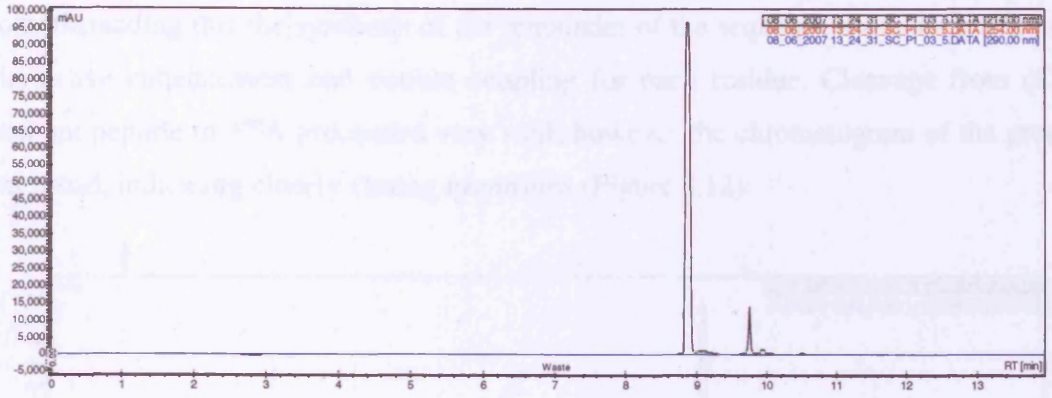


Figure 3.11a – Chromatogram of crude Cram[32-46]

Sequence: CIIIPGATCPGDYAN

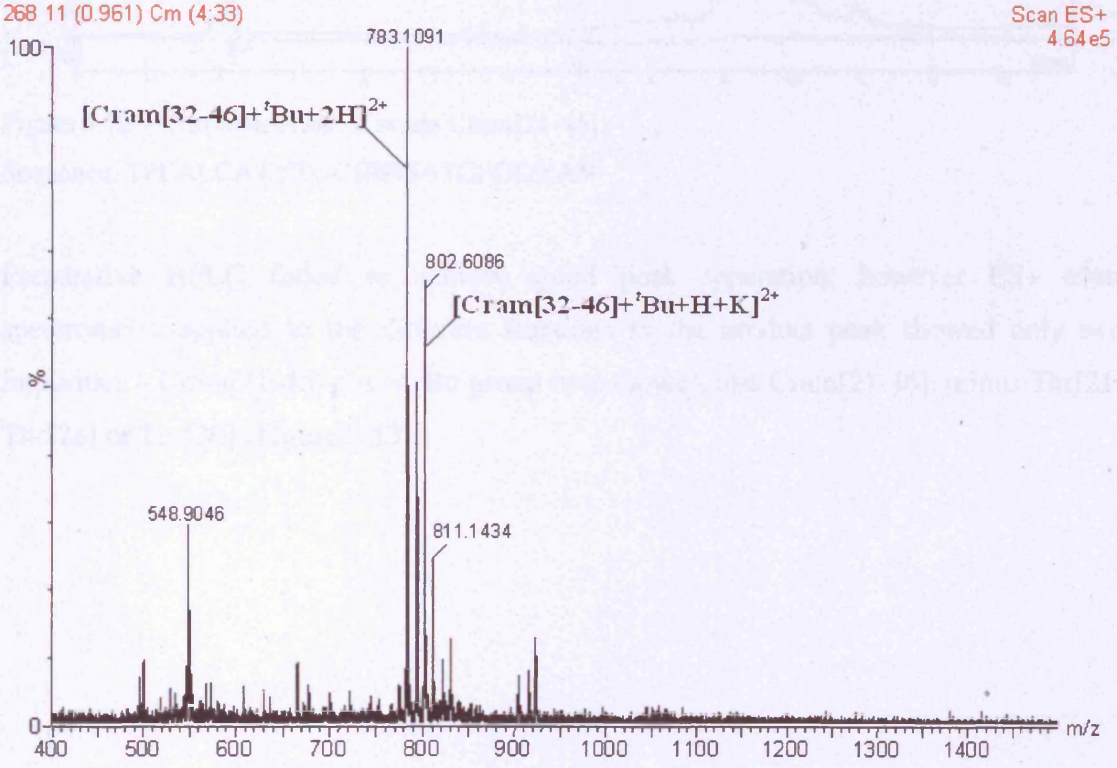


Figure 3.11b – MS spectrum of crude Cram[32-46]

Calculated m/e for [(CIIIPGATCPGDYAN)+H]$^+$: 1507.692

The fact that effective coupling of the Ile[34]-Ile[33]-Cys[32] portion proceeded *without* the MWPS was counter-intuitive as we had anticipated that microwave irradiation would reduce hydrophobic interactions and hence improve the coupling efficiency.

Notwithstanding this the synthesis of the remainder of the sequence was attempted, using microwave enhancement and double coupling for each residue. Cleavage from of the resultant peptide in TFA proceeded very well; however the chromatogram of the product was broad, indicating closely eluting impurities (Figure 3.12).

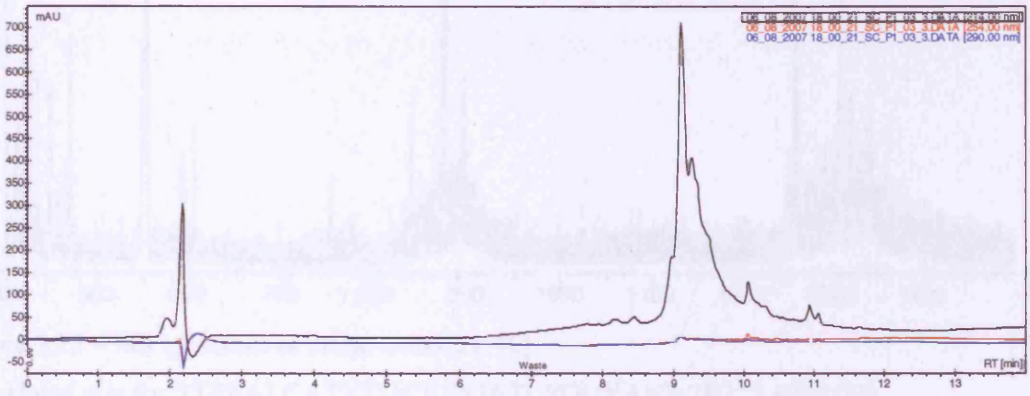


Figure 3.12 – Chromatogram of crude Cram[21-46]
Sequence: TPEALCATYTGCIIIPGATCPGDYAN

Preparative HPLC failed to achieve good peak separation; however ES+ mass spectrometry applied to the different fractions in the product peak showed only two impurities – Cram[21-46] plus a ^{t}Bu group (not shown), and Cram[21-46], minus Thr[21], Thr[28] or Thr[30] (Figure 3.13).

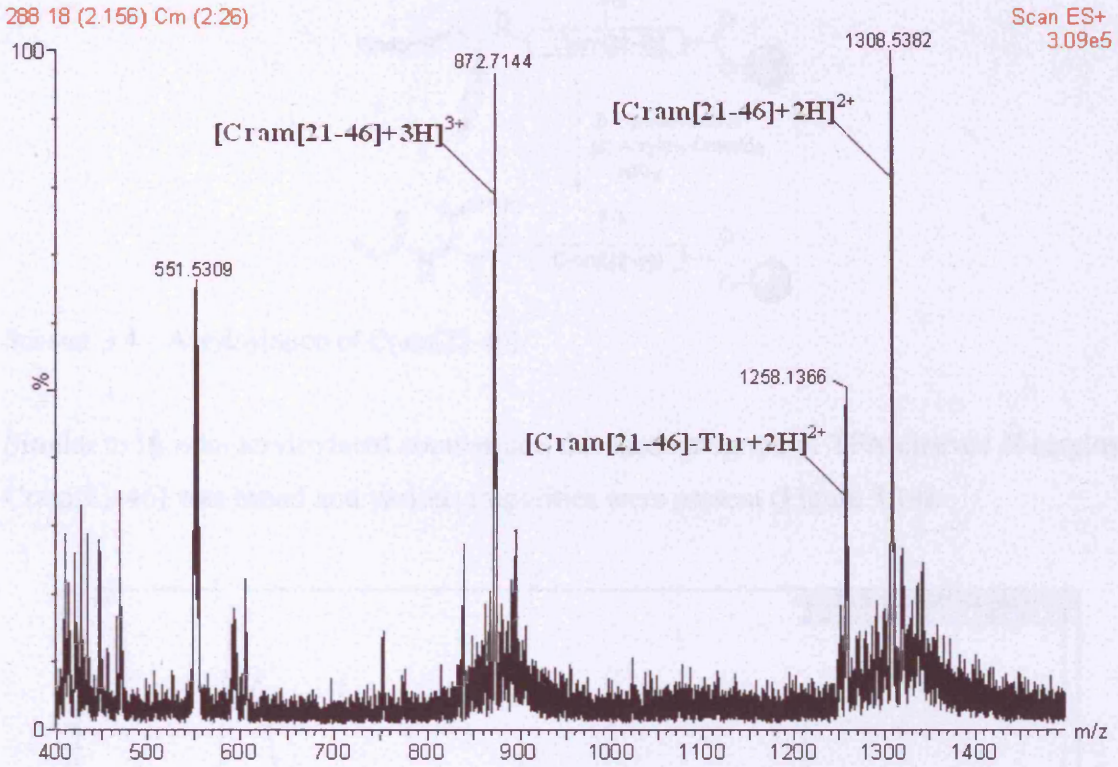


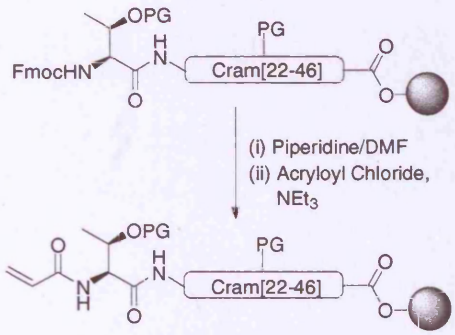
Figure 3.13 – MS spectrum of crude Cram[21-46]

Calculated m/e for $[(TPEALCATYTGCIIIPGATCPGDYAN)+2H]^{2+}$: 1308.095

These impurities, however, both had low intensities relative to the product peak. The ^{t}Bu-Cram[21-46] impurity observed in the ES+ mass spectrum could result from several different compounds with different ^{t}Bu points of attachment. This could account for the broadness of the chromatogram.

3.3.2.2 – N-Acryloyl Cram[21-46]

A portion of Cram[21-46] was acryloylated according to Scheme 3.4.



Scheme 3.4 – Acryloylation of Cram[21-46]

Similar to its non-acryloylated counterpart, the chromatogram of TFA-cleaved *N*-acryloyl Cram[21-46] was broad and various impurities were present (Figure 3.14).

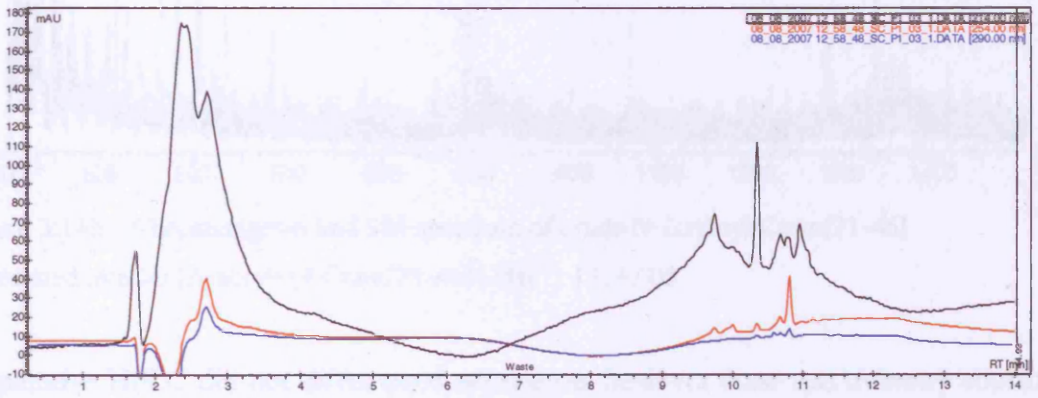


Figure 3.14a – Chromatagram of crude *N*-acryloyl Cram[21-46]

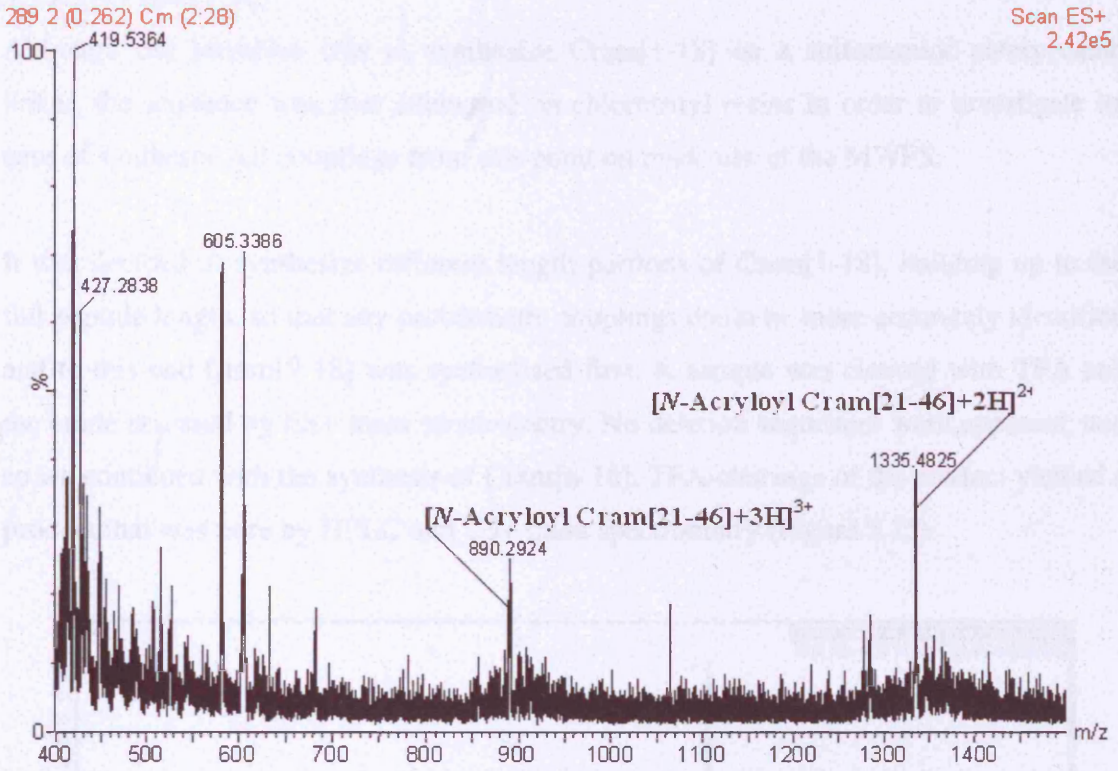


Figure 3.14b – Chromatagram and MS spectrum of crude *N*-acryloyl Cram[21-46]
Calculated m/e for [*N*-acryloyl Cram[21-46]+2H]$^{2+}$: 1334.605

Preparative HPLC did not effect good separation, however mass spectrometry confirmed acryloylation had taken place (Figure 3.14); both by presence of the *N*-acryloyl Cram[21-46] in several fractions, and total absence of the Cram[21-46] peak in any fraction.

In conclusion, despite the ambiguously broad chromatogram peaks, we observed no evidence for deletion sequences in the ES+ mass spectrum. ES+ mass spectrometry, furthermore, provides definitive evidence that *N*-acryloyl Cram[21-46] has been made. We therefore believe with optimization *N*-acryloyl Cram[21-46] can be produced in high purity. The efficiency of the acetic acid cleavage was not addressed and this will be of key importance to the future application of the methodology.

3.3.2.3 – Cram[1-18]

Although our intention was to synthesize Cram[1-18] on a sulfonamide safety catch linker, the sequence was first attempted on chlorotrityl resins in order to investigate its ease of synthesis. All couplings from this point on made use of the MWPS.

It was decided to synthesize different length portions of Cram[1-18], building up to the full peptide length, so that any problematic couplings could be more accurately identified and to this end Cram[9-18] was synthesized first. A sample was cleaved with TFA and the crude assessed by ES+ mass spectrometry. No deletion sequences were apparent, and so we continued with the synthesis of Cram[6-18]. TFA-cleavage of the product yielded a product that was pure by HPLC and ES+ mass spectrometry (Figure 3.15).

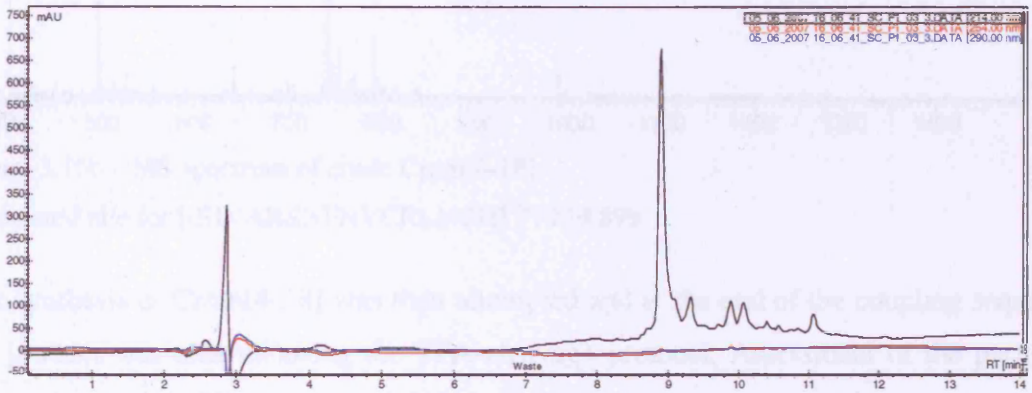


Figure 3.15a – Chromatogram of crude Cram[6-18]

Sequence: SIVARSNFNVCRL

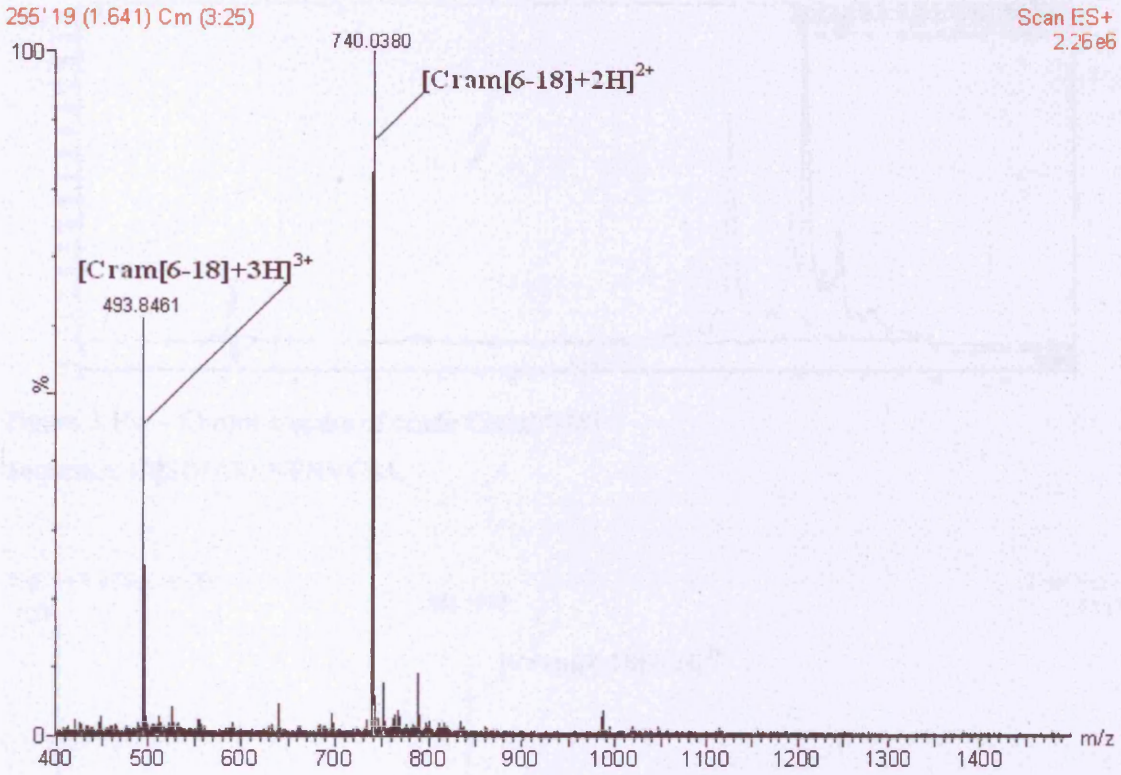


Figure 3.15b – MS spectrum of crude Cram[6-18]

Calculated m/e for [(SIVARSNFNVCRL)+2H] $^{2+}$: 739.899

The synthesis of Cram[4-18] was then attempted and at the end of the coupling sequence the product was cleaved using the TFA-cleavage protocol. Assessment of the purity of the product using HPLC indicated that the desired product was present but it was impure (Figure 3.16). ES+ mass spectrometry seemed to indicate that the peptide was present but it was contaminated with a residual trityl-bearing peptide. In order to obtain pure material we carried out preparative HPLC, and were able to obtain a pure sample of the desired peptide.

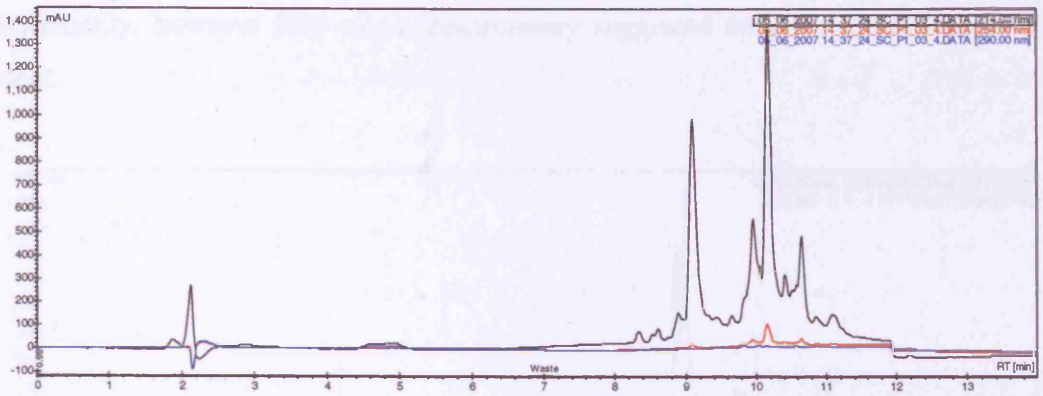


Figure 3.16a – Chromatogram of crude Cram[4-18]

Sequence: CPSIVARSNFNVCRL

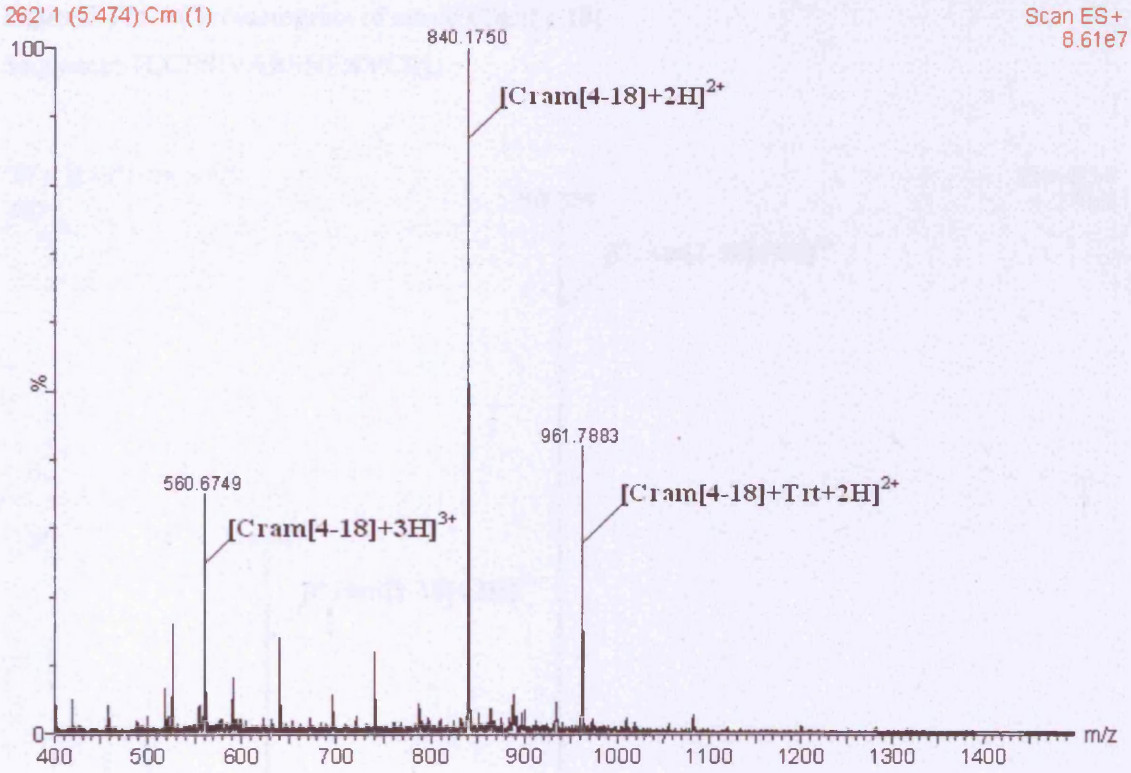


Figure 3.16b – MS spectrum of crude Cram[4-18]

Calculated m/e for [(CPSIVARSNFNVCRL)+2H] $^{2+}$: 839.930

Finally, both Cram[2-18] and Cram[1-18] were synthesized. HPLC analysis of TFA-cleaved Cram[2-18] revealed that there were two peptides present (Figure 3.17),

unfortunately, however ES+ mass spectrometry suggested only the desired peptide was present.

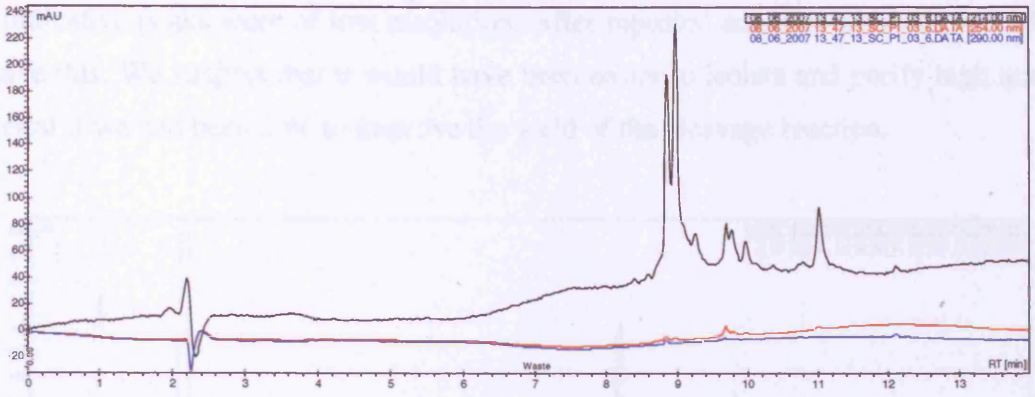


Figure 3.17a – Chromatogram of crude Cram[2-18]

Sequence: TCCPSIVARSNFNVCRL

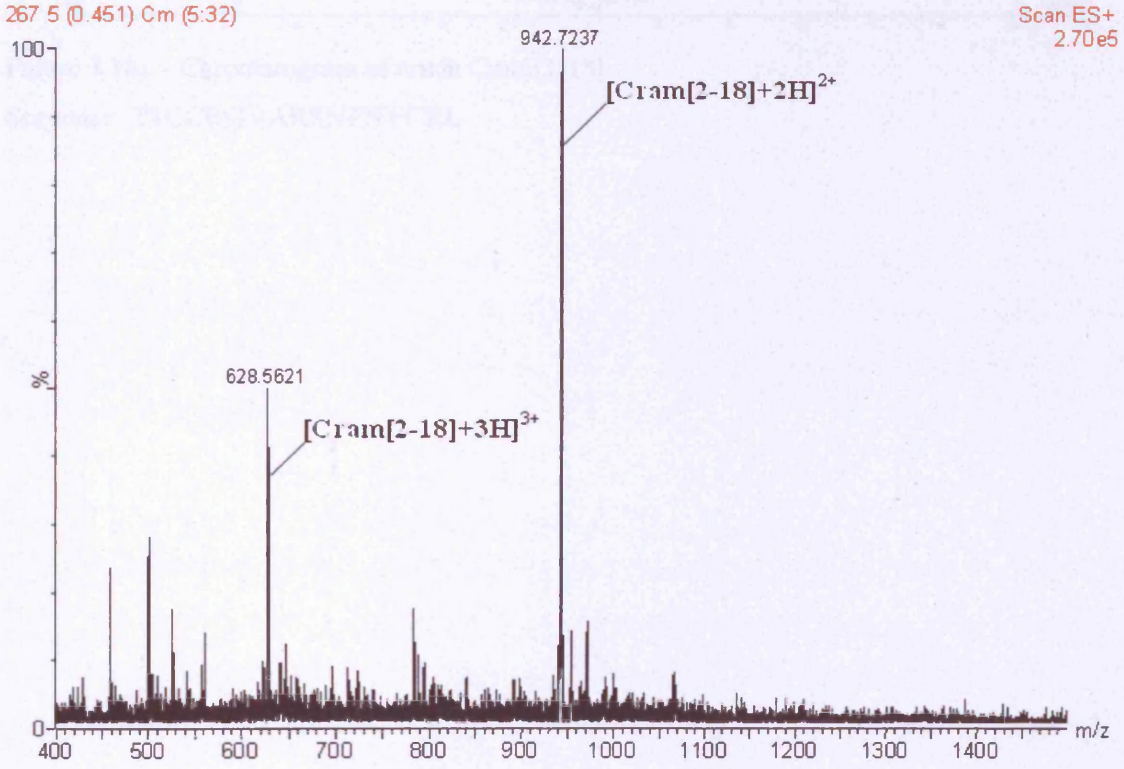


Figure 3.17b – MS spectrum of crude Cram[2-18]

Calculated m/e for [(TCCPSIVARSNFNVCRL)+2H] $^{2+}$: 941.958

Cram[1-18] appeared to be significantly less pure upon examination (Figure 3.18). Moreover, the TFA-cleavage was poor yielding, furnishing very small quantities of the desired material. The ES+ mass spectrum indicated the presence of Cram[1-18], although the indicative peaks were of low resolution. After repeated attempts we were not able to resolve this. We suspect that it would have been easier to isolate and purify high quality material if we had been able to improve the yield of the cleavage reaction.

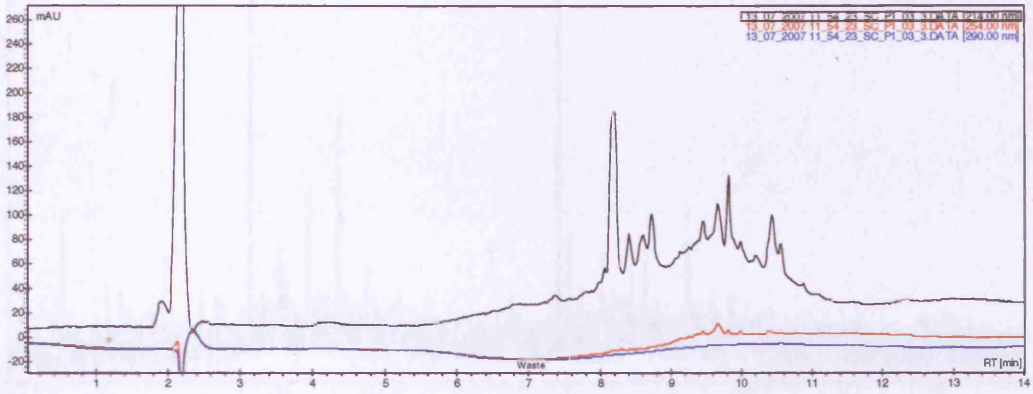


Figure 3.18a – Chromatogram of crude Cram[1-18]

Sequence: TTCCPSIVARSNFNVCRL

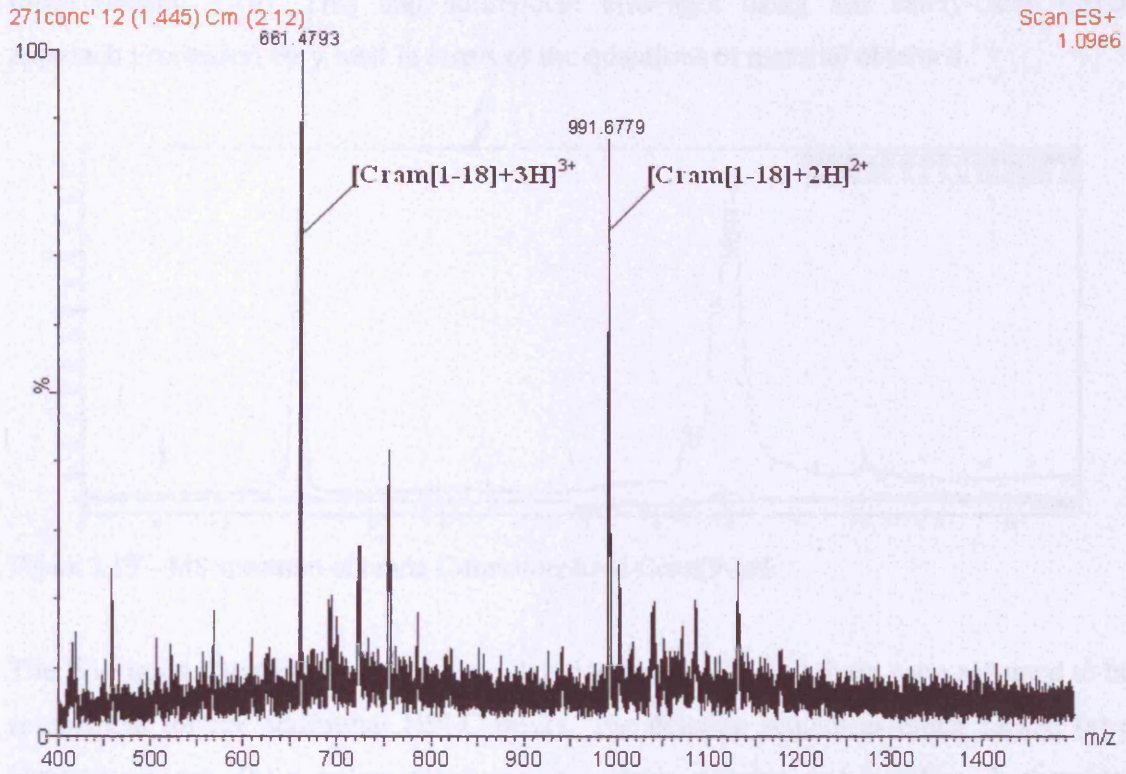


Figure 3.18b – MS spectrum of crude Cram[1-18]

Calculated m/e for $[(TTCCPSIVARSNFNVCRL)+2H]^{2+}$: 992.482

Despite the potential limitations of our cleavage protocol and its apparent deterioration with increasingly complex peptides, we decided not to attempt to optimize this protocol but rather to work with the safety-catch linker as this had always been envisaged for the total chemical synthesis.

3.3.2.4 – Synthesis of C-functionalized Cram[9-18]

It was decided to first explore the synthesis and cleavage of Cram[9-18] on a safety catch linker. SPPS, with double coupling of appropriate residues (see Section 3.3.1) was carried out, and the sulfonamide activated using iodoacetonitrile. Cleavage and *C*-functionalization was then achieved in one step using homoallylamine, and TFA deprotection of the cleaved peptide afforded the functionalized peptide product. HPLC analysis, however, showed there to be several peptide products with similar retention

times (Figure 3.19). This and subsequent cleavages using this safety-catch linker approach proceeded very well in terms of the quantities of material obtained.

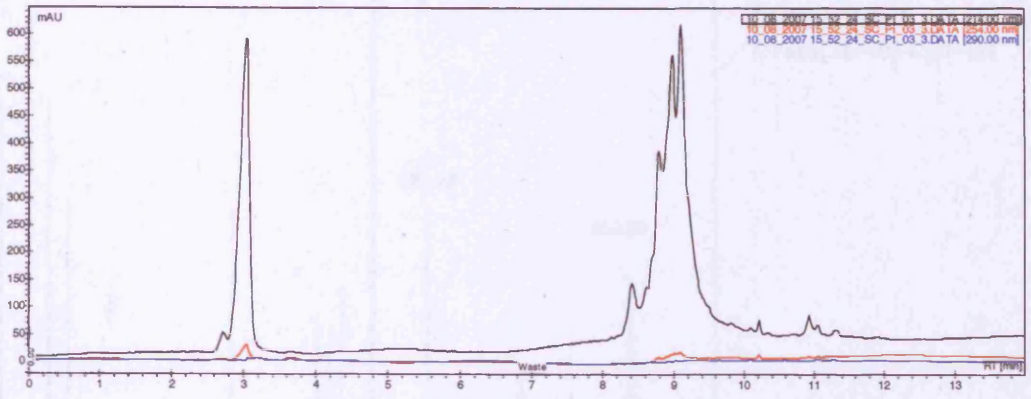


Figure 3.19 – MS spectrum of crude *C*-functionalized Cram[9-18]

The ES+ mass spectrum revealed three deletion sequences, and these were assumed to be responsible for the additional HPLC peaks. The deletion sequences either lacked (a) a cysteine residue, (b) a valine residue or (c), both cysteine and a valine. It therefore seemed sensible to repeat the SPPS of this sequence, doubling the coupling of the valine and cysteine residues. Hence we repeated the sequence but with this modified coupling regime, however once again, the cleaved, *C*-functionalized and deprotected material was impure by HPLC (Figure 3.20).

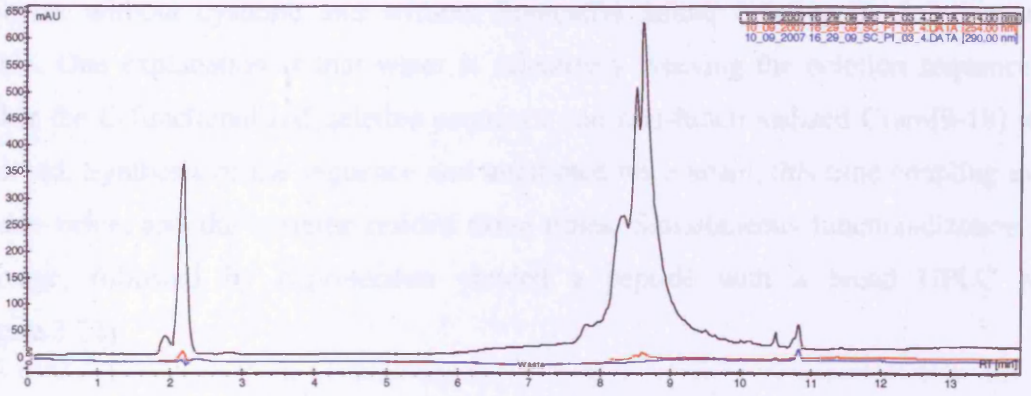


Figure 3.19 – Chromatogram of crude *C*-functionalized Cram[9-18]

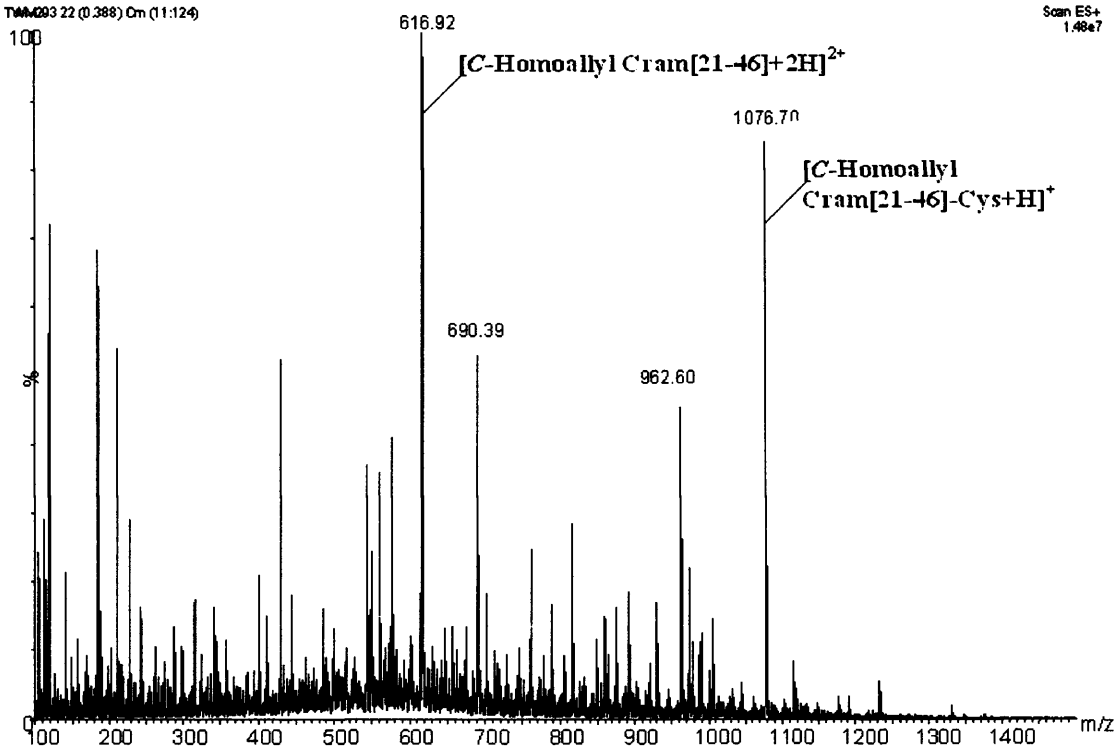


Figure 3.20 – MS spectrum of crude *C*-functionalized Cram[9-18]

Calculated m/e for [C-Homoallyl Cram[9-18]+2H]$^{2+}$: 616.834

The ES+ mass spectrum indicated that doubling of the valine and cysteine residues did eliminate the previous deletion sequences, inasmuch as they were not visible. Interestingly, however, a new impurity exhibiting a mass which corresponds to the sequence without cysteine and without homoallyl amine functionalization was now visible. One explanation is that water is selectively cleaving the deletion sequence, as neither the *C*-functionalized deletion sequence and non-functionalized Cram[9-18] were observed. Synthesis of the sequence was attempted once again, this time coupling every residue twice, and the cysteine residue *three* times. Simultaneous functionalization and cleavage, followed by deprotection yielded a peptide with a broad HPLC peak (Figure 3.21).

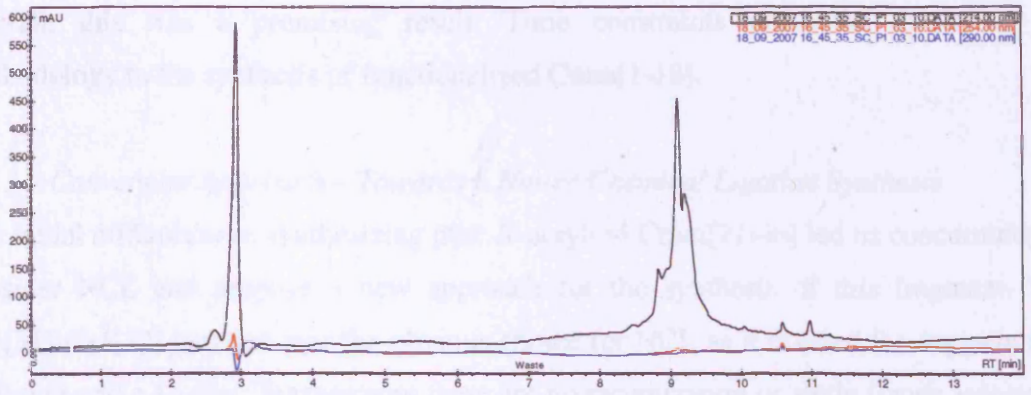


Figure 3.21a – Chromatogram of crude *C*-functionalized Cram[9-18]

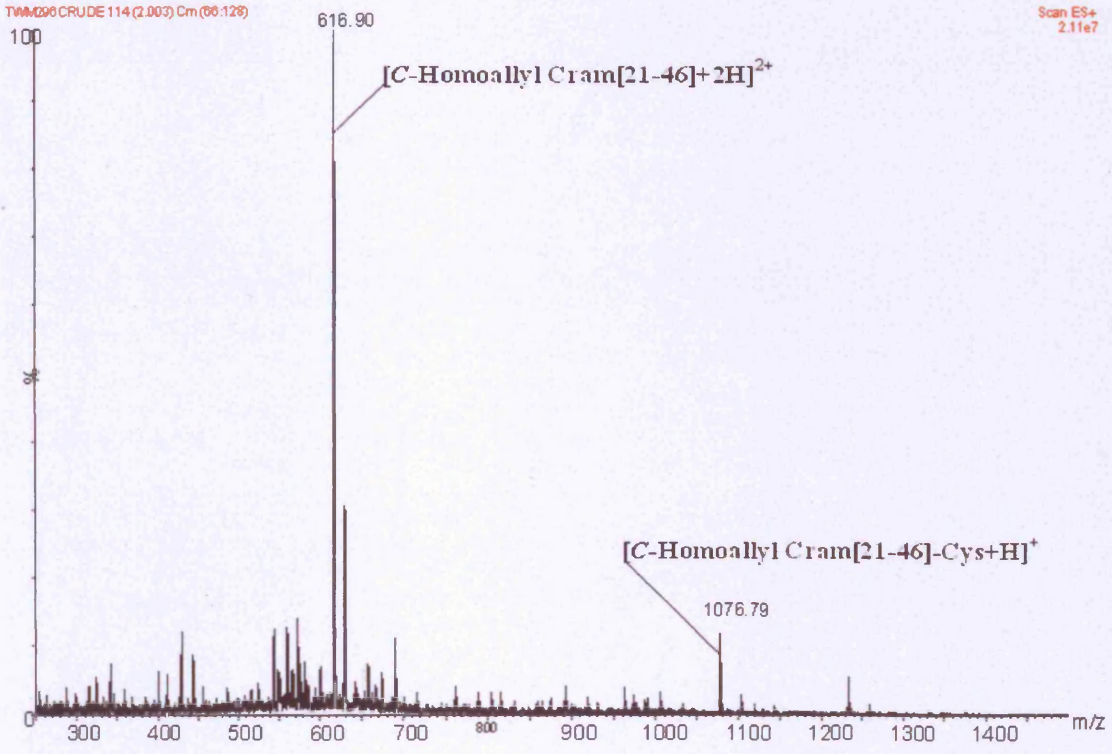


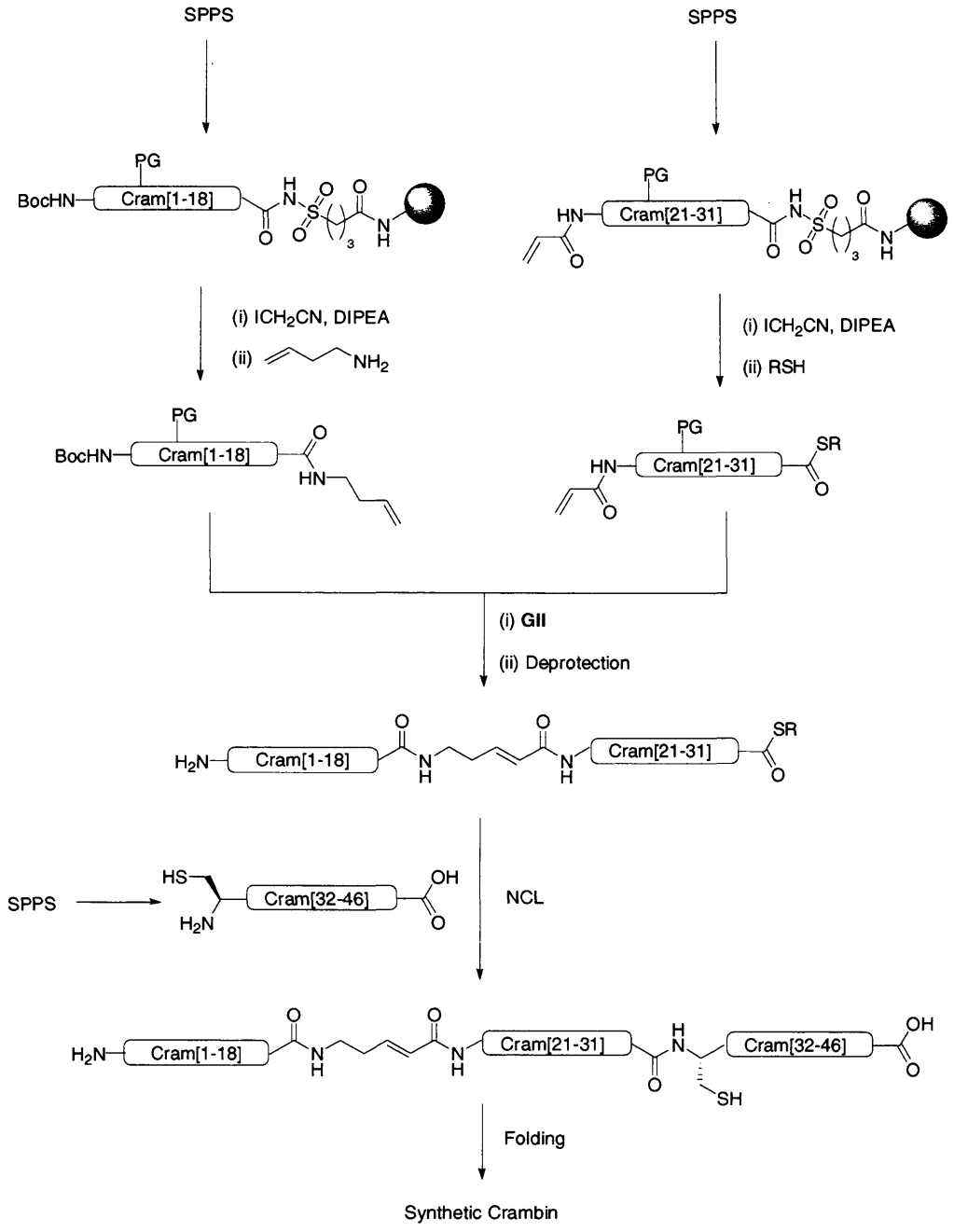
Figure 3.21b – MS spectrum of crude *C*-functionalized Cram[9-18]

Examination of the ES+ mass spectrum showed the desired functionalized sequence as the dominant peak. The peak corresponding to the sequence lacking cysteine and the homoallyl amine functionalization was still visible, but was significantly less intense. This indicated the triple coupling of the cysteine residue had mostly resolved this problem.

Overall, this was a promising result. Time constraints prevented extending the methodology to the synthesis of functionalized Cram[1-18].

3.3.3 – Convergent Approach – Towards a Native Chemical Ligation Synthesis

The initial difficulties in synthesizing pure *N*-acryloyl Cram[21-46] led us concurrently to consider NCL and propose a new approach for the synthesis of this fragment. The Gly[31]-Cys[32] junction was the obvious choice for NCL as it divided the segment into a 10-mer and a 14-mer. Furthermore there are no racemization or steric issues associated with glycine. The proposed application of NCL to the synthesis of Crambin is illustrated in Scheme 3.5.



Scheme 3.5 – Proposed use of native chemical ligation

We had already synthesized Cram[32-46] pure (Section 3.3.2.1), and so all that was needed was the *N*-acryloyl Cram[21-31] thioester.

3.3.3.1 – Synthesis of Cram[21-31]

Cram[21-31] was synthesized on chlorotrityl resins to gain an idea of the accessibility of this sequence. This proceeded excellently with double coupling of each residue, and cleavage with TFA yielded a product visibly clean by HPLC (Figure 3.22).

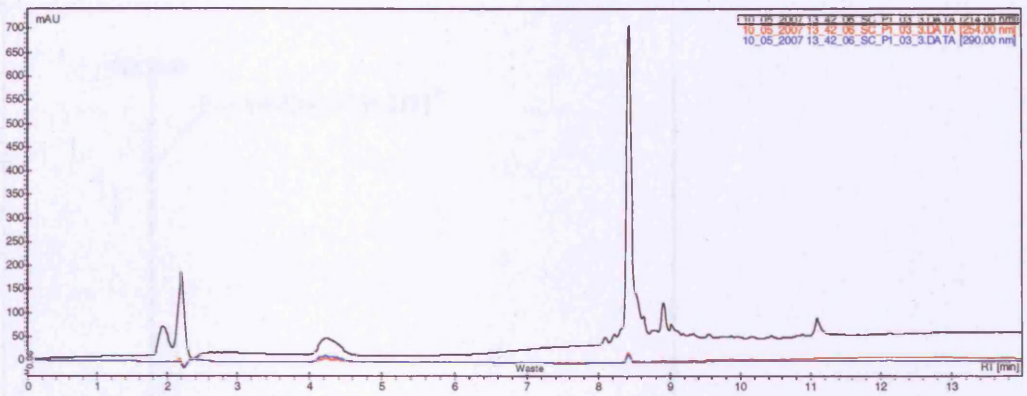


Figure 3.22a – Chromatogram of crude Cram[21-31]

Sequence: TPEALCATYTG

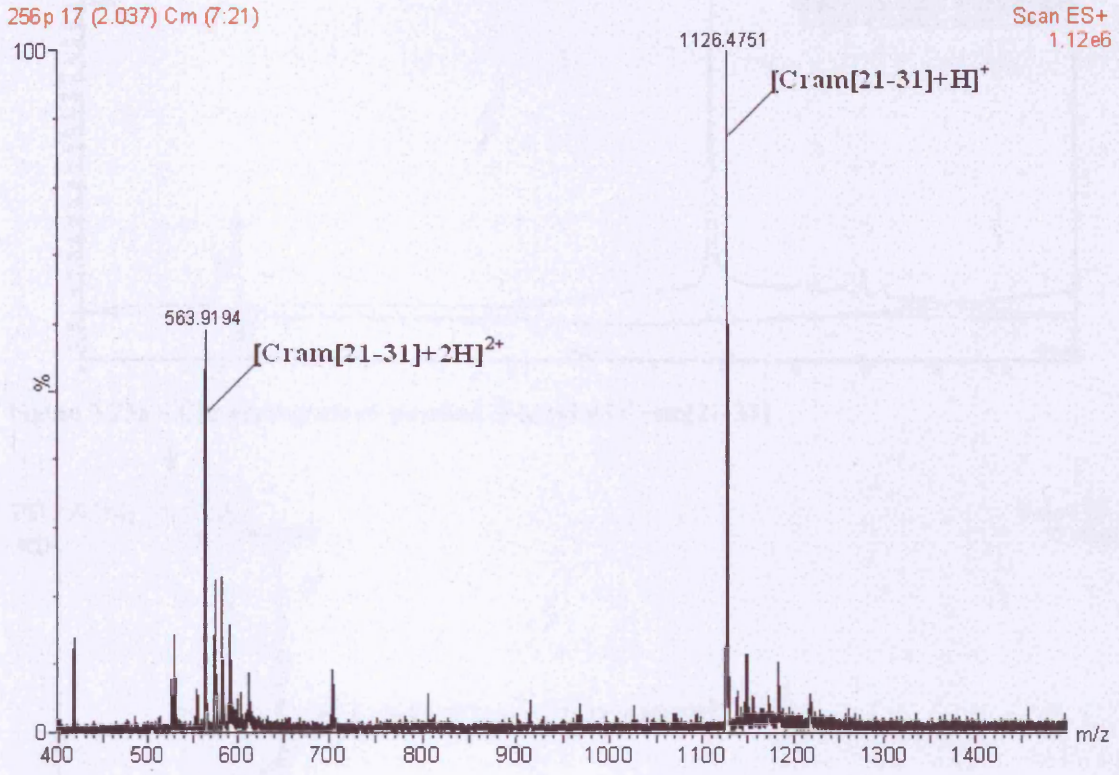


Figure 3.22b – MS spectrum of crude Cram[21-31]

Calculated m/e for [(TPEALCATYTG)+H]$^+$: 1126.509

A portion of the resins were deprotected and acryloylated as described previously (Scheme 3.4). Cleavage with TFA yielded the impure product (Figure 3.22), preparative HPLC afforded the pure acryloylated product (Figure 3.23).

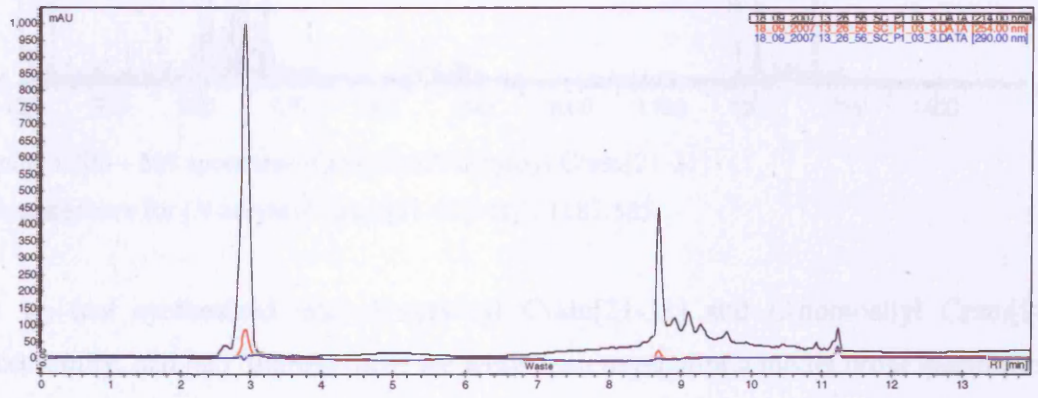


Figure 3.22 – Chromatogram of crude *N*-acryloyl Cram[21-31]

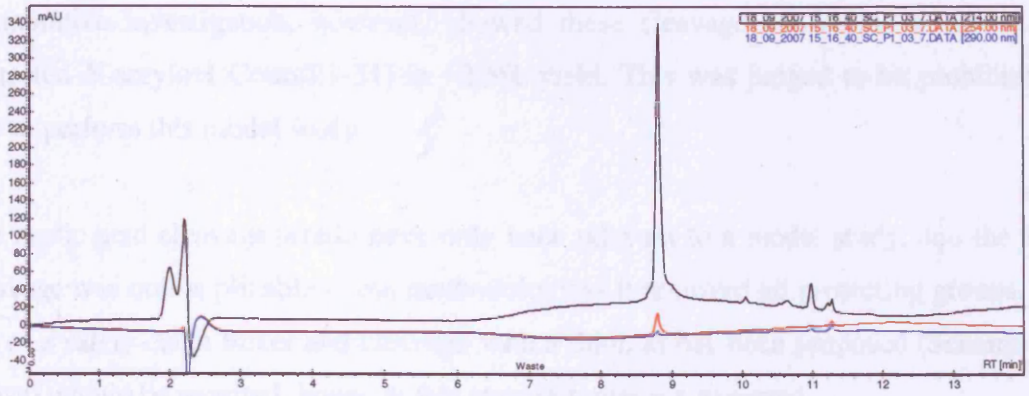


Figure 3.23a – Chromatogram of purified *N*-acryloyl Cram[21-31]

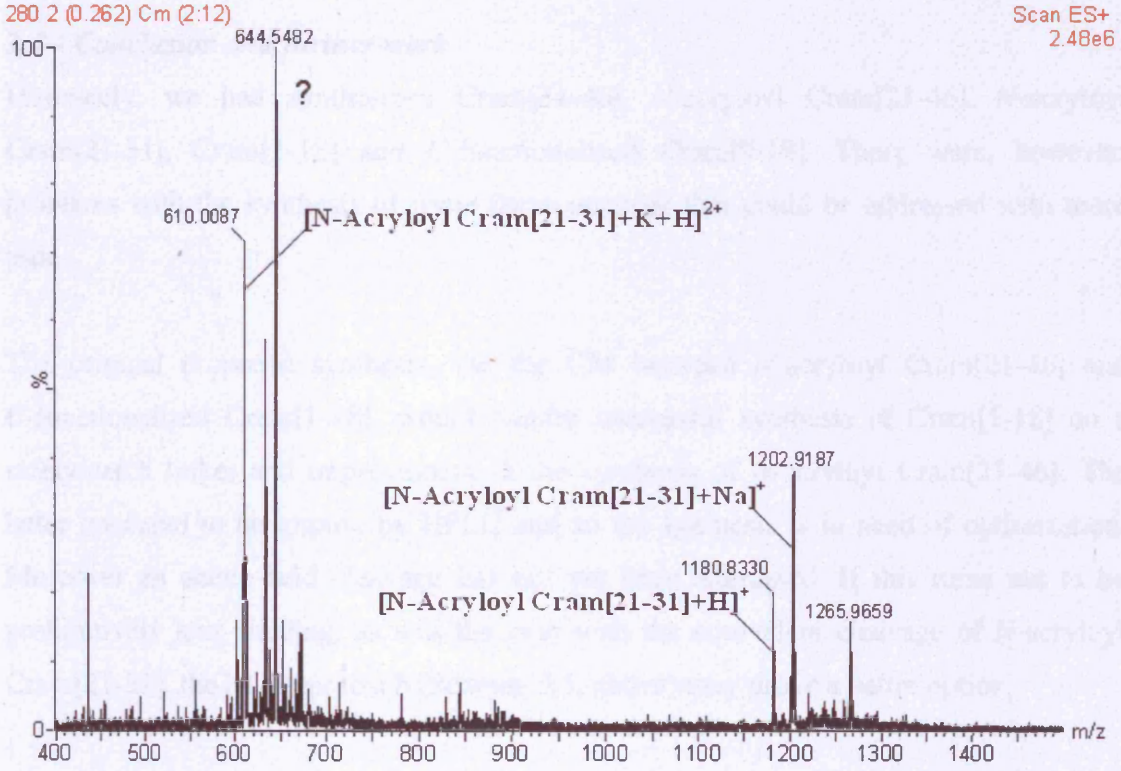


Figure 3.23b – MS spectrum of purified *N*-acryloyl Cram[21-31]

Calculated m/e for [*N*-acryloyl Cram[21-31]+H]$^+$: 1181.535

As we had synthesized both *N*-acryloyl Cram[21-31] and *C*-homoallyl Cram[9-18] successfully, and had limited time, we were keen to attempt a model cross metathesis on these segments. In order for *N*-acryloyl Cram[21-31] to be cleaved with protecting groups intact, it was necessary to investigate the efficiency of an acetic acid cleavage.

Quantitative investigation, however, showed these cleavage conditions to afford the protected *N*-acryloyl Cram[21-31] in ~2.5% yield. This was judged to be prohibitively low to perform this model study.

The acetic acid cleavage would have only been relevant to a model study, and the TFA cleavage was non-applicable to our methodology as it removed all protecting groups. The use of a safety-catch linker and cleavage with a thiol, as has been proposed (Scheme 3.5, above), would be required, however this approach was not explored.

3.4 – Conclusion and further work

Ultimately, we had synthesized Cram[31-46], *N*-acryloyl Cram[21-46], *N*-acryloyl Cram[21-31], Cram[1-18] and *C*-functionalized Cram[9-18]. There were, however, problems with the synthesis of some these peptides that could be addressed with more time.

The original proposed synthesis, *via* the CM between *N*-acryloyl Cram[21-46] and *C*-functionalized Cram[1-18], would require successful synthesis of Cram[1-18] on a safety-catch linker and improvement of the synthesis of *N*-acryloyl Cram[21-46]. The latter appeared to be impure by HPLC and so the synthesis is in need of optimization. Moreover an acetic acid cleavage has not yet been attempted. If this turns out to be prohibitively low yielding, as was the case with the equivalent cleavage of *N*-acryloyl Cram[21-31], the NCL approach (Scheme 3.5, above) may prove a better option.

In order for NCL to be employed successfully, Cram[32-46] is required, unprotected. This has been successfully achieved. Furthermore, the synthesis of *N*-acryloyl Cram[21-31], has been achieved. Its synthesis on and thiol cleavage from the sulfonamide safety catch linker, however, needs to be explored. Given our previous success with safety catch linkers in the synthesis of *C*-functionalized Cram[9-18] we are hopeful this approach will prove fruitful.

Both proposed syntheses require *C*-functionalized Cram[1-18]. Although *C*-functionalized Cram[9-18] exhibited a broad peak by HPLC, ES+ evidence suggests it is of reasonable purity. Given the broadness of the peak, however, and the difficulties encountered in the synthesis of Cram [1-18] on chlorotrityl resins, it is very likely synthesis of *C*-functionalized Cram[1-18] will be non-trivial.

Chapter 4 – Experimental

4.1 – Synthetic Organic Chemistry

4.1.1 – General information

All reactions were carried out under an argon atmosphere, using oven-dried glassware. All chemicals were purchased from commercial sources and were used as received. Peptide coupling reactions were monitored in some cases using a Micromass Platform LC instrument. Acryloylations and thermal cross metathesis reactions were monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates (254 mm) and in some cases using a using a Micromass Platform LC instrument. Microwave reactions utilized a CEM Discover instrument unless otherwise stated. All yields quoted are isolated yields. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C at 75 MHz on a Bruker AMX 300 at ambient temperature unless otherwise stated. The chemical shifts for ^{1}H and ^{13}C are quoted in ppm relative to residual protiated signals of the solvent. Coupling constants (*J*) are given in Hertz. Mass spectra were obtained using electrospray ionization on a VG70-SE or a MAT 900 XP mass spectrometer using a triple quadrupole mass analyser. Infrared spectra were obtained using a solution cell on a Shimadzu FTIR 8700 Spectrophotometer or using a Perkin Elmer Spectrum 100 (solid). Optical rotations were measured using monochromatic light at 589 nm with a 1 dm cell and either a model AA-1000 polarimeter, at 294 K or a Polaar 2000 polarimeter at ambient temperatures. Melting points were measured, where appropriate, with a Gallenkamp apparatus and are uncorrected.

A note on the assignment of ^{1}H NMR – Superscript a and b distinguish between diastereotopic protons on the same carbon and thus are not intended to uniquely define protons within the same molecule.

Abbreviations

s	singlet	br	broad
d	doublet	q	quartet
t	triplet	app	apparent
		obsc	obscured

4.1.2 – General procedures

Protocol I – *C*-functionalization of amino acids with homoallyl amine

Protected amino acid (3.4 mmol) and HOBt (455 mg, 3.4 mmol) were dissolved in DCM (15 mL) and the mixture stirred vigorously. EDC.HCl (1.2 g, 6.8 mmol) and DIPEA (1.18 mL, 6.8 mmol) were added and the mixture stirred for 10 minutes. Homoallyl amine or the corresponding HCl salt (3.4 mmol) was added and the resultant mixture stirred for 4 h. The mixture was then diluted with DCM (15 mL), and washed with HCl (2x10 mL, 0.5 M), $NaHCO_3$ (2x10 mL, 0.5 M), water (2x10 mL) and saturated brine (10 mL) and dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate).

Protocol II – *N*-Acryloylation of amino acid with acryloyl chloride

To a stirred solution of amino acid ester (1.7 mmol) and Et_3N (0.5 mL, 3.7 mmol) in DCM (10 mL) was added acryloyl chloride (2 mmol, as a 1 M solution in DCM) was over 1 h. The resultant mixture was allowed to warm to RT and stirred for a further 18 h. The DCM was removed *in vacuo*, and ethyl acetate added (80 mL). The solid was filtered off and the filtrate was washed with HCl (1x10 mL, 0.5 M), $NaHCO_3$ (2x10 mL, 0.5 M), water (1x10 mL) and saturated brine (1x10 mL), dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate).

Protocol III – Thermal CM between *N*-acryloyl and *C*-homoallyl amine functionalized amino acids

To a stirred solution of *N*-acryloyl amino acid (0.2 mmol) and *C*-homoallyl amine functionalized amino acid (1.3 equivalents) in DCM (3 mL) was added **GII** (7-19 mol% in 2 mL DCM). The resultant mixture was heated to reflux and stirred until the *N*-acryloyl amino acid was no longer visible by TLC. The reaction mixture was allowed to

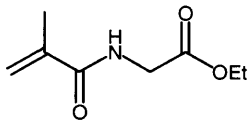
cool to RT, and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate).

Protocol IV – Optimized microwave cross metathesis

A solution of *N*-acryloyl amino acid (0.1 mmol), *C*-homoallyl amine functionalized amino acid (1.3 equivalents) and **GII** (18 mol%) in DCM (0.5 mL) was sealed in a microwave vial. The solution was then subjected to microwave irradiation (300 W, 100 °C) for 30 mins. The reaction mixture was then degassed with argon for approximately 30 seconds, and the mixture and subjected to microwave irradiation (300 W, 100 °C) for a further 30 mins. The reaction mixture was allowed to cool to RT, and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate).

4.1.3 – Synthesis of Compounds in Number Order

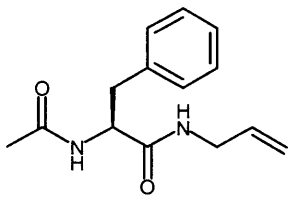
3 – (2-Methyl-acryloylamino)-acetic acid ethyl ester



To a stirred solution of methacrylic acid (2.5 mL, 29 mmol) in dichloromethane (100 mL) was added HOBt (4.0 g, 30 mmol), EDC.HCl (5.2 g, 29 mmol), DIPEA (5.3 mL, 29 mmol) and glycine ethyl ester hydrochloride, **2**, (2.13 g, 15.2 mmol). The reaction mixture was stirred at RT for 6½ h. The reaction mixture was washed with HCl (1x20 mL, 0.5 M), sodium bicarbonate solution (1x20 mL, 0.5 M) and once with saturated brine (1x20 mL) and dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate). The product was obtained as a colourless viscous liquid (2.52 g, 97 %).

^{1}H NMR (DMSO d_6): 8.34 (t, J = 6.0, 1 H, N*H*); 5.72 (s, 1 H, C=C*H cis*); 5.46 (s, 1 H, C=C*H trans*); 4.08 (q, J = 7.0, 2 H, OCH_2CH$_3$); 3.83 (d, J = 6.0, 2 H, NCH_2); 1.86 (s, 3 H, C=CCH_3); 1.81 (t, J = 7.0, 3 H, OCH$_2$CH_3). ^{13}C NMR (DMSO d_6) : 169.7 (C=O); 167.7 (C=O); 139.2 (C); 119.7 (CH$_2$); 60.2 (CH$_2$); 40.9 (CH$_2$); 18.3 (CH$_3$); 13.9 (CH$_3$).
IR, solution cell, DCM (cm^{-1}): 3348 (N-H stretch); 2984; 2937; 1749 (C=O stretch); 1666 (C=O stretch); 1622; 1531.
HRMS calc. for $C_8H_{13}NO_3$: 194.0788 $[M+Na]^+$; Found 194.0793.

5 – (S)-2-Acetylamino-*N*-allyl-3-phenyl-propionamide[128]



To a stirred solution of **4** (1.0 g, 4.8 mmol) in DCM was added HOBt (0.65 g, 4.8 mmol), DIPEA (0.88 mL, 4.8 mmol) and EDC.HCl (1.7 g, 4.8 mmol), and the mixture stirred at RT for 10 minutes. Allyl amine (1.42 mL, 19.2 mmol) was added and the resultant mixture stirred at RT for 6 h. The reaction mixture was washed once with HCl (20 mL, 0.5 M), once with sodium bicarbonate solution (20 mL, 0.5 M) and once with saturated brine (20 mL) and dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo* affording **5** as a white solid (0.88 g, 75%).

^{1}H NMR (DMSO d_6): 8.15 (t, J = 6.0, 1 H, N*H*); 8.13 (d, J = 8.5, 1 H, N*H*); 7.15-7.28 (m, 5 H, Ar-*H*); 5.72 (ddt, J = 5.0, J = 17.0, J = 10.5, 1 H, NHCH$_2$C*H*); 4.98-5.08 (m, 2 H, C=CH_2); 4.47 (ddd, J = 9.5, J = 8.5, J = 5.0, 1 H, NC*H*CO); 3.66 (m, 2 H, NCH_2); 2.95 (dd, J = 5.0, J = 13.5, 1 H, NCHCH^b*H*^{a}); 2.74 (dd, J = 9.5, J = 13.5, 1 H, NCHCH^a*H*^{b}); 1.75 (s, 3 H, COCH_3). ^{13}C NMR (DMSO d_6): 171.0 (C=O); 169.0 (C=O); 138.0 (C); 135.0 (CH); 129.0 (CH); 128.0 (CH); 126.1 (CH); 114.9 (CH$_2$); 54.1 (CH); 40.7 (CH$_2$); 37.8 (CH$_2$); 22.4 (CH$_3$).

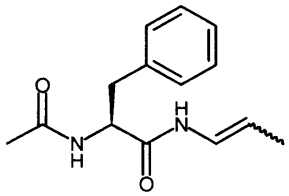
IR, solid disc (cm^{-1}): 3251 (N-H stretch); 3061; 1670 (C=O stretch); 1635; 1551.

$(\alpha)_D$ -4.7° (c = 1.0, MeCN).

HRMS calc. for $C_{14}H_{18}N_2O_2$: 269.1266 $[M+Na]^+$; Found 269.1263.

Mp: 136-139 °C. Lit. 160-161 °C.

7 – (S)-2-Acetylamino-3-phenyl-*N*-prop-2-enyl-propionamide



To a stirred solution of **5** (100 mg, 0.41 mmol) and **3** (70 mg, 0.40 mmol) in DCM (12 mL) was added **HG** (14 mg, 5 mol%). The mixture refluxed for 20 h, by which time **5** was no longer visible by TLC. The reaction mixture was allowed to cool to RT, and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) yielding **7** (94 mg, 94%) as a mixture of isomers (*Cis*: *trans*, 22:25).

Cis product –

^{1}H NMR (DMSO d_6): 9.45 (d, J = 10.5, 1 H, N*H*); 8.21 (d, J = 8.0, 1 H, N*H*); 7.18-7.27 (m, 5 H, Ar-*H*); 6.50 (dq, J = 10.5, J = 1.5, 1 H, NC*H*=C); 4.65-4.75 (m, 2 H, NC*H*CO, C=C*H*Me superimposed); 2.91 (dd, J = 5.0, J = 13.5, 1 H, NCHCH^b*H*^{a}); 2.76 (dd, J = 10.0, J = 13.5, 1 H, NCHCH^a*H*^{b}); 1.76 (s, 3 H, COCH_3); 1.60 (dd, J = 7.0, J = 1.5, 1 H, C=CCH_3). ^{13}C NMR (DMSO d_6) – 169.7 (C=O); 169.2 (C=O); 137.7 (C); 129.1 (CH); 127.9 (CH); 126.2 (CH); 122.0 (CH); 105.7 (CH); 53.6 (CH); 37.6 (CH_2); 22.3 (CH_3); 11.3 (CH_3).

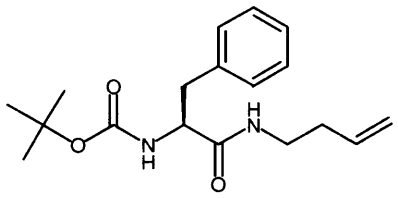
Trans product –

^{1}H NMR (DMSO d_6): 9.83 (d, J = 10.0, 1 H, N*H*CH=CH); 8.19 (d, J = 8.5, 1 H, N*H*); 7.14-7.29 (m, 5 H, Ar-*H*); 6.54 (ddd, J = 14.0, J = 10.0, J = 2.0, 1 H, NC*H*=C); 5.18 (dq, J = 14.0, J = 6.5, 1 H, C=C*H*Me); 4.41-4.49 (m, 1 H, NC*H*CO); 2.92 (dd, J = 5.0, J = 13.5, 1 H, NCHCH^b*H*^{a}); 2.73 (dd, J = 9.5, J = 13.5, 1 H, NCHCH^a*H*^{b}); 1.75 (s, 3 H, COCH_3); 1.60 (dd, J = 6.5, J = 2.0, 1 H, C=CCH_3). ^{13}C NMR (DMSO d_6): 169.1 (C=O); 168.6 (C=O); 137.7 (C); 129.0 (CH); 128.0 (CH); 126.2 (CH); 123.6 (CH); 107.1 (CH); 54.0 (CH); 37.5 (CH_2); 22.3 (CH_3); 14.8 (CH_3).

IR, solid disc (cm^{-1}): 3266 (N-H stretch); 3065; 1666 (C=O stretch); 1636; 1528; 1549.

HRMS calc. for $C_{14}H_{18}N_2O_2$: 269.1266 [M+Na]$^+$; Found 269.1274.

11 – (S)-(1-but-3-enylcarbamoyl-2-phenyl-ethyl)-carbamic acid tert-butyl ester[93]



BocPheOH (1.7g, 6.4 mmol) was *C*-functionalized with homoallyl amine hydrochloride according to protocol I yielding **11** as a waxy solid (1.3g, 64%).

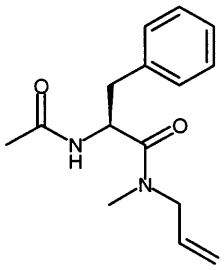
^{1}H NMR ($CDCl_3$): 7.17-7.29 (m, 5 H, Ar-*H*); 5.99 (br, 1 H, N*H*); 5.61 (app ddt, J = 17.0, J = 10.5, J = 6.5, 1 H, H_2CC*H*=CH_2); 5.20 (br, 1 H, N*H*); 4.91-4.98 (m, 2 H, H_2CCH=CH_2); 4.29 (app q, J = 7.0, 1 H, NHC*H*); 3.13 (m, 2 H, NHCH_2); 3.01 (m, 2 H, CHCH_2); 2.02-2.16 (m, 2 H, CH_2CH=CH_2); 1.38 (s, 9 H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$): 171.1 (C=O); 155.4 (C=O); 136.9 (C); 134.9 (CH); 129.3 (CH); 128.6 (CH); 126.9 (CH); 117.1 (CH_2); 80.0 (C); 56.0 (CH); 38.9 (CH_2); 38.4 (CH_2); 33.4 (CH_2); 28.3 (CH_3).

HRMS calc. for $C_{19}H_{26}N_2O_3$: 319.2022 [M+H]$^+$; found 319.2027.

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 2978; 2934; 1709 (C=O stretch); 1676 (C=O stretch).

$(\alpha)^{25}_D$ +4.9 (*c* 0.50, $CHCl_3$)

13 – (S)-2-Acetylamino-*N*-allyl-*N*-methyl-3-phenyl-propionamide



To a stirred solution of **4** (0.60 g, 2.9 mmol) in DCM (20 mL) was added *N*-Methyl allylamine (0.41 mL, 4.4 mmol), HOBt (0.39 g, 2.9 mmol), DIPEA (0.96 mL, 5.8 mmol) and EDC.HCl (1.0 g, 5.8 mmol), and the mixture stirred at RT for 1 h. The reaction mixture was washed with HCl (2x10 mL, 0.5 M), sodium bicarbonate solution (2x10 mL, 0.5 M) and saturated brine (2x10 mL) and dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo* yielding **13** as a viscous, colourless liquid (0.65 g, 86%).

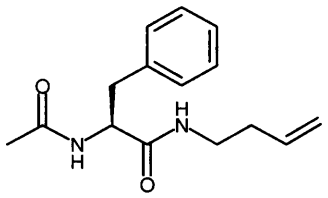
^{1}H NMR, 400 MHz, 383 K (DMSO d_6): 7.7 (br, N*H*); 7.18-7.27 (m, Ar-*H*); 5.61-5.71 (m, CH_2CH=C); 5.03-5.10 (m, C=CH_2); 4.94 (q, NHC*H*); 2.98 (m, NCHCH^aH^b); 2.82 (m, NCHCH$^aH^b$), 2.80 (s, NCH_3); 1.80 (s, COCH_3). ^{13}C NMR (DMSO d_6): 171.5, 171.2 (C=O); 169.1 (C=O); 138.1, 137.9 (CH); 133.9, 133.3 (CH); 129.6 (CH); 128.6 (CH); 126.8 (CH); 116.9, 116.7 (CH_2); 51.5, 50.4 (CH); 50.2, 49.7 (CH_3); 38.6, 38.0 (CH_2); 34.7, 33.6 (CH_2); 22.8 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3296 (N-H stretch); 3061; 3032; 2982; 2930; 1636 (C=O stretch); 1541.

HRMS calc. for $C_{15}H_{20}N_2O_2$: 283.1417 $[M+Na]^+$; Found 283.1417.

$(\alpha)_D$ -1.2° (c = 0.62, MeCN).

18 – (S)-2-Acetylamino-*N*-but-3-enyl-3-phenyl-propionamide



4 (0.70 g, 3.4 mmol) was *C*-functionalized with homoallyl amine HCl according to protocol I. The crude product was recrystallized from ethyl acetate and petroleum ether yielding **18** as white crystals (0.82 g, 93%).

^{1}H NMR (DMSO d_6): 8.11 (d, J = 9.0, 1 H, N*H*CH); 7.98 (t, J = 5.5, 1 H, N*H*CH$_2$); 7.14-7.27 (m, 5 H, Ar-*H*); 5.70 (app ddt, J = 10.5, J = 17.0, J = 7.0, 1 H, H$_2$CC*H*=CH$_2$); 4.95-5.05 (m, 2 H, CH=C*H*$_2$); 4.43 (app dt, J = 9.0, J = 5.5, 1 H, NHC*H*); 2.95-3.17 (m, 2 H, NHC*H*$_2$); 2.92 (dd, J = 5.5, J = 13.5, C*H*^{a}H^{b}Ph); 2.72 (dd, J = 9.0, J = 13.5, CH^a*H*^{b}Ph); 2.08 (app q, J = 7.0, 2 H, *H*$_2$CCH=CH$_2$); 1.75 (s, 3 H COCH$_3$). ^{13}C NMR (MeOH d_4): 173.5 (C=O); 173.0 (C=O); 138.5 (C); 136.5 (CH); 130.3 (CH); 129.4 (CH); 127.8 (CH); 117.0 (CH$_2$); 56.3 (CH); 39.8 (CH$_2$); 39.2 (CH$_2$); 34.6 (CH$_2$); 22.4 (CH$_3$).

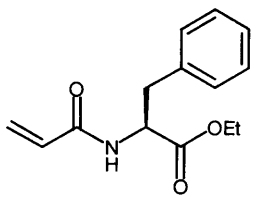
IR, solution cell, DCM (cm^{-1}): 3423 (N-H stretch); 3310 (N-H stretch); 2932; 1665 (C=O stretch); 1504.

HRMS calc. for $C_{15}H_{20}N_2O_2$: 283.1422 $[M+Na]^+$; found 283.1423.

$(\alpha)_D$ +5.6° (c = 0.31, MeCN).

Mp: 127-128 °C.

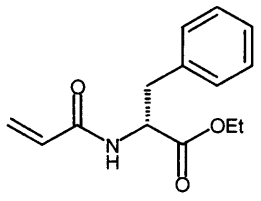
19 – (S)-2-Acryloylamino-3-phenyl-propionic acid ethyl ester[129]



PheOEt HCl (3.0 g, 13 mmol) was *N*-acryloylated according to protocol II. The product was purified by two recrystallizations from ethyl acetate and petroleum ether yielding **19** as white, needle-like crystals (78%).

^{1}H NMR (MeOH d_4): 7.17-7.29 (m, 5 H, Ar-*H*); 6.26 (dd, *J* = 9.5, *J* = 17.0, 1 H, H_2CCH); 6.17 (dd, *J* = 2.5, *J* = 17.0, 1 H, HC=C*H*H *trans*); 5.64 (dd, *J* = 2.5, *J* = 9.5, 1 H, HC=C*H*H *cis*); 4.70 (dd, *J* = 6.0, *J* = 8.5, 1 H, HNC*H*); 4.12 (app q, *J* = 7.0, 2 H, OCH_2); 3.15 (dd, *J* = 6.0, *J* = 14.0, 1 H, CH^aH^bPh); 2.99 (dd, *J* = 8.5, *J* = 14.0, 1 H, CH^aH^bPh); 1.18 (app t, *J* = 7.0, 3 H, CH_2CH_3). ^{13}C NMR (MeOH d_4): 173.0 (C=O); 167.9 (C=O); 138.1 (C); 131.4 (CH); 130.2 (CH); 129.5 (CH); 127.9 (CH); 127.4 (CH_2); 62.4 (CH_2); 55.5 (CH); 38.5 (CH_2); 14.4 (CH_3).
IR, solution cell, DCM (cm^{-1}): 3420 (N-H stretch); 2986; 2359; 1735 (C=O stretch); 1676 (C=O stretch); 1632; 1506.
HRMS calc. for $C_{14}H_{17}NO_3$: 270.1106 $[M+Na]^+$; found 270.1112.
$(\alpha)^{25}_D$ +133.6 (*c* 0.24, $CHCl_3$). Lit. $(\alpha)^{20}_D$ 121.7° (*c* = 3.0, C_6H_6).
Mp: 64-66 °C. Lit. 66°C

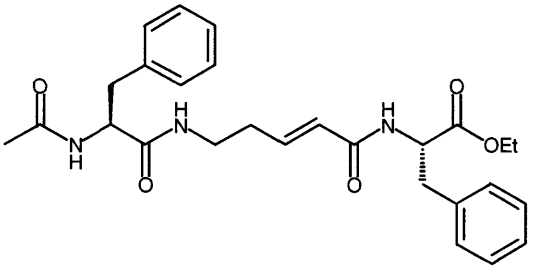
(R)-2-Acryloylamino-3-phenyl-propionic acid ethyl ester



***R*-19**

To a stirred solution of D-Phe (1.0 g, 6.1 mmol) in ethanol at -5°C was added thionyl chloride (0.50 mL, 6.7 mmol). This was allowed to warm to RT and the mixture stirred for a further 18 h. The solvent was removed *in vacuo* and the crude mixture *N*-acryloylated according to protocol II. ***R*-19** was obtained as a white solid (0.48 g, 32%).
NMR and IR data analogous to **19**
$(\alpha)^{25}_D$ –132.0 (*c* 0.27, $CHCl_3$)

20 – (S)-2-[5-((S)-2-Acetylamino-3-phenyl-propionylamino)-pent-2-enoylamino]-3-phenyl-propionic acid ethyl ester



Method 1: To a stirred solution of **18** (240 mg, 0.92 mmol) and **19** (230 mg, 0.40 mmol) in DCM (3 mL) was added **GII** (40 mg, 5 mol%). The mixture was refluxed for 24 h, by which time a white precipitate had formed. DCM (5 mL) was added and the mixture refluxed for a further hour to achieve dissolution. The solution was allowed to cool to RT, silica gel (5 g) added and the solvent removed *in vacuo*. Purification of the dry-loaded product mixture using silica-gel chromatography (ethyl acetate, then 5% MeOH in ethyl acetate) afforded **20** as a light brown solid (269 mg, 61%). Repetition using **18** (120 mg, 0.46 mmol), **19** (100 mg, 0.40 mmol) and **GII** (23 mg, 7 mol%) afforded 174 mg **20** (90%) and the homodimer, **21** (33 mg, 15%).

Method 2: A solution of **18** (33 mg, 1.3 mmol), **19** (25 mg, 0.10 mmol) and **GII** (16 mg, 19 mol%) in DCM (1.0 mL) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 90°C) for 1 h. The solution was allowed to cool to RT, silica gel (1.5 g) added and the solvent removed *in vacuo*. Purification of the dry-loaded product mixture using silica-gel chromatography (ethyl acetate, then 5% MeOH in ethyl acetate) gave **32** as a pale brown solid (34 mg, 71%).

^{1}H NMR (DMSO d_6): 8.37 (d, J = 6.0, 1 H, N*H*); 8.11 (d, J = 9.0, 1 H, N*H*); 8.06 (br, 1 H, N*H*); 7.14-7.28 (m, 10 H, Ar-*H*); 6.61 (app dt, J = 15.5, J = 7.0, 1 H, CH_2CH=CH); 5.95 (d, J = 15.5, 1 H, CH_2CH=C*H*); 4.49 (app dt, J = 8.0, J = 6.0, 1 H, NHC*H*); 4.41 (app dt, J = 9.0, J = 5.0, 1 H, NHC*H*); 4.02 (app q, J = 7.0, 2 H, OCH_2); 2.86-3.20 (m, 4 H, $CHCH_2$Ph, $NHCH_2$ superimposed); 2.91 (dd, J = 5.0, J = 13.5, 1 H, CH^aH^bPh); 2.71

(dd, J = 9.0, J = 13.5, 1 H, CH^aH^bPh); 2.19 (app q, J = 7.0, 2 H, H_2CCH=CH); 1.74 (s, 3 H, $COCH_3$); 1.08 (app t, 3 H, J = 7.0, CH_2CH_3).

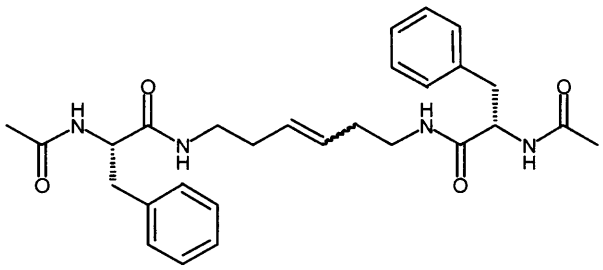
^{13}C NMR (DMSO d_6): 171.6 (C=O); 171.1 (C=O); 168.9 (C=O); 164.6 (C=O); 140.6 (CH); 138.0 (C); 137.1 (C); 129.0 (CH); 128.95 (CH); 128.2 (CH); 127.9 (CH); 126.5 (CH); 126.1 (CH); 124.9 (CH); 60.4 (CH_2); 54.0 (CH); 53.6 (CH); 37.8 (CH_2); 37.5 (CH_2); 36.8 (CH_2); 31.4 (CH_2); 22.4 (CH_3); 13.9 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3424 (N-H stretch); 2963; 2358; 1734 (C=O stretch); 1674 (C=O stretch); 1506.

HRMS calc. for $C_{27}H_{33}N_3O_5$: 502.2318 [M+Na]$^+$; found 502.2325.

$(\alpha)^{16}_D$ -4.6 (c 0.33, $CHCl_3$).

21 – (S)-2-Acetylamino-N-[6-((S)2-acetylamino-3-phenyl-propionylamino)-hex-3-enyl]-3-phenyl-propionamide



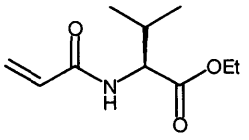
^{1}H NMR (DMSO d_6): 8.10 (d, J = 9.0, 2 H, NHCH); 7.93 (t, J = 5.5, 2 H, NHCH$_2$); 7.13-7.26 (m, 10 H, Ar-H); 5.26-5.33 (m, 2 H, CH=CH); 4.43 (app dt, J = 5.5, J = 9.0, 2 H, NHCH); 2.95-3.14 (m, 4 H, NHCH_2); 2.91 (dd J = 5.5, J = 13.5, 2 H, CH^aH^{b}Ph); 2.71 (dd, J = 13.5, J = 9.0, 2 H, CH^aH^bPh); 1.95-2.03 (m, 4 H, CH_2CH=CH); 1.74 (s, 6 H, COCH_3).

^{13}C NMR (DMSO d_6): 171.0 (C=O); 169.0 (C=O); 138.0 (C); 129.1 (CH); 128.7 (CH); 128.0 (CH); 126.2 (CH); 54.1 (CH); 38.4 (CH_2); 38.0 (CH_2); 32.2 (CH_2); 22.5 (CH_2).

IR, solid disc (cm^{-1}): 3279 (N-H stretch); 2929; 1639 (C=O stretch); 1533.

HRMS calc. for $C_{28}H_{36}N_4O_4$: 515.2629 [M+Na]$^+$; found 515.2636.

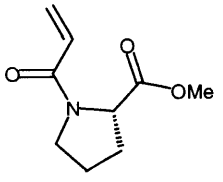
22 – (S)-2-Acryloylamino-3-methyl-butyric acid ethyl ester[129]



ValOEt HCl (1.7 g, 9.4 mmol) was acryloylated according to protocol II, yielding **22** as a pale yellow liquid (1.0 g, 54%).

^{1}H NMR (MeOH d_4): 6.40 (dd, J = 10.0, J = 17.0, 1 H, H_2CCH); 6.24 (dd, J = 2.0, J = 17.0, 1 H, HC=C*H*H *trans*); 5.68 (dd, J = 2.0, J = 10.0, 1 H, HC=C*H*H *trans*); 4.37 (d, J = 6.5, 1 H, HNC*H*); 4.14-4.23 (m, 2 H, OCH_2); 2.16 (app octet, J = 6.5, 1 H, $CH(CH_3)_2$); 1.26 (app t, J = 7.0, OCH_2CH_3); 0.96 (d, J = 6.5, 6 H, $CH(CH_3)_2$). ^{13}C NMR (MeOH d_4): 173.0 (C=O); 168.2 (C=O); 131.5 (CH); 127.5 (CH_2); 62.2 (CH_2); 59.4 (CH); 31.8 (CH); 19.5 (CH_3); 18.5 (CH_3); 14.5 (CH_3).
IR, solution cell, DCM (cm^{-1}): 3678; 3425 (N-H stretch); 2972; 1732 (C=O stretch); 1678 (C=O stretch); 1626; 1510.
HRMS calc. for $C_{10}H_{17}NO_3$: 222.1106 $[M+Na]^+$; found 222.1104.
$(\alpha)^{16}_D$ +19.7 (*c* 1.24, $CHCl_3$). Lit. $(\alpha)^{20}_D$ -9.9 (*c* 9.9, C_6H_6).

23 – (S)-1-acryloyl-pyrrolidine-2-carboxylic acid methyl ester[130]



ProOMe HCl (415 mg, 2.5 mmol) was acryloylated according to protocol II yielding **23** as a colourless liquid (340 mg, 72%). This consisted of a 3:1 mix of rotamers, clearly visible by NMR. Heating did not result in coalescence of peaks and thus the spectrum for each conformation is reported separately.

Conformation 1 (75%)

^{1}H NMR ($CDCl_3$, 400 MHz): 6.44 (dd, *J* = 10.0, *J* = 17.0, 1 H, C*H*=CH_2); 6.35 (dd, *J* = 2.0, *J* = 17.0, 1 H, CH=C*H*$_2$); 5.67 (dd, *J* = 10.0, *J* = 2.0, 1 H, CH=C*H*$_2$); 4.51 (dd, *J* = 4.0, *J* = 8.5, 1 H, NC*H*); 3.69 (s, 3 H, OMe); 3.55-3.77 (m, 2 H, NC*H*$_2$); 1.91-2.27 (m, 4 H, NCH2C*H*$_2$C*H*$_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 172.6 (C=O); 164.4 (C=O); 128.4 (CH_2); 128.0 (CH); 58.8 (CH_3); 52.1 (CH); 46.9 (CH_2); 29.1 (CH_2); 24.7 (CH_2).

Conformation 2 (25%)

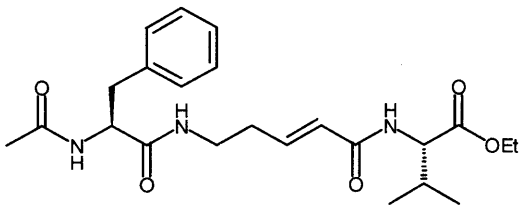
^{1}H NMR ($CDCl_3$, 400 MHz): 6.33 (dd, *J* = 2.0, *J* = 17.0, 1 H, CH=C*H*$_2$); 6.20 (dd, *J* = 10.5, *J* = 17.0, 1 H, CH=CH_2); 5.62 (dd, *J* = 2.0, *J* = 10.5, 1 H, CH=C*H*$_2$); 4.48 (dd, *J* = 3.0, *J* = 8.5, 1 H, NC*H*); 3.70 (s, 3 H, OMe); 3.55-3.77 (m, 2 H, NC*H*$_2$); 1.91-2.27 (m, 4 H, NCH$_2$C*H*$_2$C*H*$_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 172.5 (C=O); 164.8 (C=O); 128.1 (CH_2); 128.0 (CH); 59.2 (CH_3); 52.5 (CH); 46.4 (CH_2); 31.3 (CH_2); 22.5 (CH_2).

IR, solution cell, DCM (cm^{-1}): 3476; 2959; 2883; 1744 (C=O stretch); 1649 (C=O stretch); 1609.

HRMS calc. for $C_9H_{13}NO_3$: 206.0793 $[M+Na]^+$; found 206.0793.

$(\alpha)^{14}_D$ -54.0 (*c* 0.84, MeCN). Lit $(\alpha)^{20}_D$ -110.0 (*c* 1.00, $CHCl_3$).

24 – (S)-2-[5-((S)-2-Acetylamino-3-phenyl-propionylamino)-pent-2-enoylamino]-3-methyl-butyric acid ethyl ester



Method 1: To a stirred solution of **18** (110 mg, 0.42 mmol) and **22** (80 mg, 0.40 mmol) in DCM (3 mL) was added **GII** (19 mg, 6 mol%). The resultant mixture was refluxed for 24 h, by which time a white precipitate had formed. DCM (5 mL) was added and the mixture refluxed until complete dissolution had occurred. The solution was allowed to cool to RT, silica gel (5 g) added and the solvent removed *in vacuo*. Purification the dry-loaded

product mixture by silica-gel chromatography (ethyl acetate, then 5% MeOH in ethyl acetate) yielded **24** as a pale brown solid (72 mg, 42%).

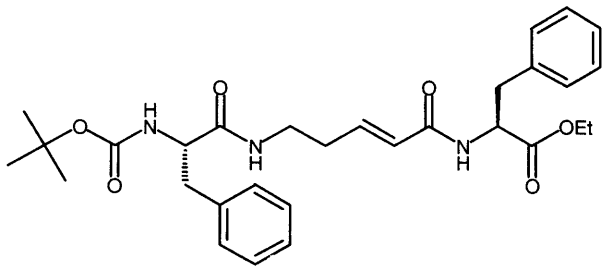
Method 2: To a stirred solution of **18** (74 mg, 0.28 mmol) and **21** (50 mg, 0.25 mmol) in DCM (3 mL) was added **GII** (21 mg, 10 mol%). The resultant mixture was refluxed for 72 h, by which time a white precipitate had formed. DCM (5 mL) was added and the mixture refluxed until complete dissolution had occurred. The solution was allowed to cool to RT, silica gel (5 g) added and the solvent removed *in vacuo*. Purification the dry-loaded product mixture by silica-gel chromatography (ethyl acetate, then 5% MeOH in ethyl acetate) yielded **25** as a pale brown solid (59 mg, 55%).

Method 3: A solution of **18** (75 mg, 0.28 mmol), **21** (52 mg, 0.25 mmol) and **GII** (16 mg, 9 mol%) in DCM (2.0 mL) and DMF (3 drops) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 90°C) for 15 min. After cooling, the mixture was irradiated for a further 15 min. The solution was allowed to cool to RT, silica gel (5 g) added and the solvent removed *in vacuo*. Purification the dry-loaded product mixture by silica-gel chromatography yielded **25** as a pale brown solid (47 mg, 55%).

^{1}H NMR (MeOH d_4): 7.16-7.32 (m, 5 H, Ar-*H*); 6.68 (app dt, J = 15.5, J = 7.0, 1 H, CH_2CH=CH); 6.07 (d, J = 15.5, 1 H, CH_2CH=C*H*); 4.52 (ddd, J = 2.0, J = 6.5, J = 9.0, 1 H, C*H*CH_2Ph); 4.35 (d, J = 6.5, 1 H, C*H*$CH(CH_3)_2$); 4.16 (app q, J = 7.0, 2 H, OCH_2); 3.21-3.31 (m, 2 H, NCH_2); 3.06 (dd, J = 6.5, J = 13.5, 1 H, C*H*^{a}H^{b}Ph); 2.85 (dd, J = 9.0, J = 13.5, 1 H CH^a*H*^{b}Ph); 2.29 (app q, J = 7.0, 2 H, NCH_2CH_2CH); 2.32 (app octet, J = 6.5, 1 H, C*H*$(CH_3)_2$); 1.89 (s, 3 H, COCH_3); 1.27 (t, J = 7.0, 3 H, CH_2CH_3); 0.94 (d, J = 6.5, 6 H, $CH(CH_3)_2$). ^{13}C NMR (MeOH d_4): 173.7 (C=O); 173.1 (C=O); 173.05 (C=O); 168.3 (C=O); 142.8 (CH); 138.5 (C); 130.3 (CH); 129.4 (CH); 127.8 (CH); 126.0 (CH); 62.2 (CH_2); 59.4 (CH); 56.4 (CH); 39.2 (CH_2); 39.1 (CH_2); 32.8 (CH_2); 31.9 (CH_3); 22.5 (CH_3); 19.5 (CH_3); 18.6 (CH_3); 14.6 (CH).
IR, solution cell, DCM (cm^{-1}): 3425 (N-H stretch); 3308 (N-H stretch); 2969; 2936; 1731 (C=O stretch); 1647 (C=O stretch); 1507.

HRMS calc. for $C_{23}H_{33}N_3O_5$: 454.2312 $[M+Na]^+$; found 454.2315.

25 – (S)-2-[(E)-5-((S)-2-*tert*-Butoxycarbonylamino-3-phenyl-propionylamino)-pent-2-enoylamino]-3-phenyl-propionic acid ethyl ester



SS-product: A solution of **11** (80 mg, 0.25 mmol), **19** (50 mg, 0.20 mmol) and **GII** (32 mg, 19 mol%) in DCM (1 mL) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 80 °C) for 1 h. The solution was allowed to cool to RT and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) affording **25** as a brown waxy solid (92 mg, 86%).

RS-product: A solution of *R*-**11** (80 mg, 0.25 mmol), **19** (50 mg, 0.20 mmol) and **GII** (32 mg, 19 mol%) in DCM (1 mL) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 80 °C) for 2 h. The solution was allowed to cool to RT and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) yielding *RS*-**25** as a brown waxy solid (71 mg, 61%).

SR-product: A solution of **11** (86 mg, 0.27 mmol), *R*-**19** (52 mg, 0.21 mmol) and **GII** (28 mg, 17 mol%) was sealed in a microwave vial. The solution was subjected to microwave irradiation (300 W, 90 °C) for 30 mins. The reaction mixture was then degassed with argon for approximately 1 min, and the mixture stirred and subjected to microwave

irradiation (300 W, 90 °C) for a further 30 mins. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) giving *SR*-**25** as a brown waxy solid (52 mg, 48%).

RR-product: **11** and **19** were reacted according to protocol IV yielding *RR*-**25** as a red waxy solid (34 mg, 63%).

SS product

^{1}H NMR ($CDCl_3$, 400 MHz): 7.14-7.28 (m, 5 H, Ph-*H*); 6.68 (br, 1 H, N*H*); 6.62 (app dt, *J* = 15.6, *J* = 7.9, 1 H, CH_2CH=CH); 6.45 (br, 1 H, N*H*); 5.64 (d, *J* = 15.6, 1 H, CH_2CH=C*H*); 5.53 (br, 1 H, N*H*); 4.90 (app td, *J* = 6.3, *J* = 7.6, 1 H, NC*H*COOEt); 4.48 (br m, 1 H, BocNC*H*); 4.16 (app q, *J* = 7.5, 2 H, OCH_2CH_3); 3.17-3.25 (br m, 2 H, $NHCH_2$); 3.06-3.17 (m, 2 H, EtO_2CHCH_2); 2.99 (d, *J* = 7.3, 2 H, $BocNHCHCH_2$); 2.10-2.30 (br m, 2 H, $NHCH_2CH_2$); 1.36 (s, 9 H, $C(CH_3)_3$); 1.22 (app t, *J* = 7.5, 3 H, OCH_2CH_3). ^{13}C NMR ($CDCl_3$): 172.2 (C=O); 171.8 (C=O); 165.1 (C=O); 155.7 (C=O); 141.1 (CH); 137.0 (C); 136.1 (C); 129.5 (CH); 129.3 (CH); 128.5 (2CH); 127.0 (CH); 126.8 (CH); 124.9 (CH); 80.0 (C); 61.5 (CH_2); 55.8 (CH); 53.9 (CH); 39.2 (CH_2); 38.1 (CH_2); 37.6 (CH_2); 31.9 (CH_2); 28.3 (CH_3); 14.1 (CH_3). Superimposable on ^{13}C NMR of *RR* product.

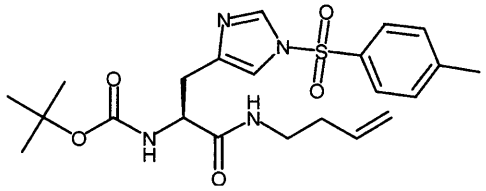
IR, solution cell, DCM (cm^{-1}): 3425 (N-H stretch); 2980; 2936; 1726 (C=O stretch); 1678 (C=O stretch); 1501.

HRMS calc. for $C_{30}H_{39}N_3O_6$: 538.2917 $[M+H]^+$; found 538.2918.

RS product

^{13}C NMR ($CDCl_3$): 171.7 (C=O); 171.3 (C=O); 164.9 (C=O); 155.4 (C=O); 141.2 (CH); 136.8 (C); 136.0 (C); 129.4 (CH); 129.3 (CH); 128.6 (CH); 128.5 (CH); 127.1 (CH); 126.9 (CH); 125.2 (CH); 80.2 (CH); 61.42 (CH_2); 56.0 (CH); 53.3 (CH); 38.8 (CH_2); 38.0 (CH_2); 37.8 (CH_2); 31.9 (CH_2); 28.3 (CH_3); 14.1 (CH_3). Superimposable on ^{13}C NMR of *SR* product.

26 – (S)-{1-but-3-enylcarbamoyl-2-[3-(toluene-4-sulfonyl)-3H-imidazol-4-yl]-ethyl}-carbamic acid tert-butyl ester[93]



BocHis(Tos)OH (654 mg, 0.48 mmol) was *C*-functionalized with homoallyl amine according to protocol I yielding **26** as a viscous colourless liquid (537 mg, 73%).

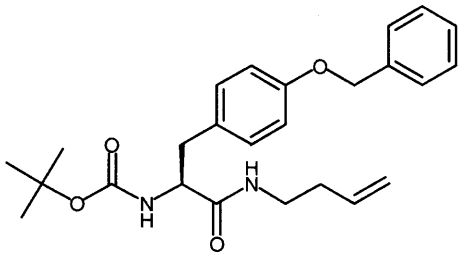
^{1}H NMR ($CDCl_3$): 8.00 (br s, 1 H, Im-*H*); 7.92 (d, *J* = 8.0, 2 H, Ts-*H*); 7.35 (d, *J* = 8.0, 2 H, Ts-*H*); 7.09 (s, 1 H, Im-*H*); 6.61 (br s, 1 H, N*H*); 5.86 (br, 1 H, N*H*); 5.63 (app ddt, 1 H, *J* = 17.5, *J* = 10.5, *J* = 6.5, H_2CCH=CH_2); 4.96-5.02 (m, 2 H, CH=CH_2); 4.40 (br s, 1 H, HNC*H*); 3.17 (app q, *J* = 6.5, 2 H, NHCH_2); 3.03 (dd, *J* = 5.5, *J* = 14.5, 1 H, CH^aH^{b}Im); 2.90 (dd, *J* = 4.5, *J* = 14.5, 1 H, CH^aH^bIm); 2.34 (s, 3 H, ArCH_3); 2.09 (app q, *J* = 6.5, 2 H, CH_2CH_2CH); 1.40 (s, 9 H, C(CH_3)$_3$). ^{13}C NMR (MeOH d_4): 173.7 (C=O); 157.5 (C=O); 148.0 (C); 141.7 (C); 138.1 (CH); 136.5 (CH); 136.2 (CH); 131.7 (CH); 128.7 (CH); 117.5 (CH_2); 116.4 (CH); 80.6 (C); 55.5 (CH); 39.8 (CH_2); 34.6 (CH_2); 31.7 (CH_2); 30.7 (CH_2); 28.7 (CH_2); 21.7 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3423 (N-H stretch); 2978; 2934; 1711 (C=O stretch); 1672 (C=O stretch); 1593.

HRMS calc. for $C_{22}H_{30}N_4O_5S$: 463.2010 $[M+H]^+$; found 463.2005.

$(\alpha)^{14}_D$ +21.2 (*c* 0.62, $CHCl_3$)

27 – [(S)-2-(4-Benzyloxy-phenyl)-1-but-3-enylcarbamoyl-ethyl]-carbamic acid tert-butyl ester



BocTyr(Bzl)OH (594 mg, 1.6 mmol) *C*-functionalized with homoallyl amine according to protocol I affording **27** as a waxy solid (417 mg, 61%).

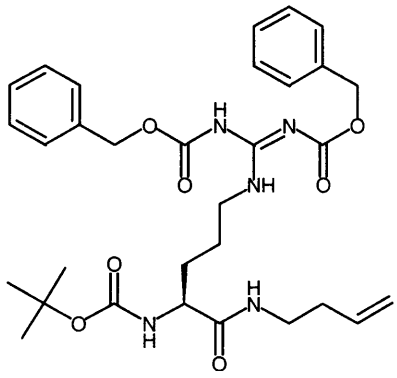
^{1}H NMR (DMSO d_6): 7.86 (app t, J = 5.5, 1 H, N*H*CH_2); 7.30-7.43 (m, 5 H, CH_2Ar-*H*); 7.13 (d, J = 8.5, 2 H, *H* meta to O); 6.89 (d, J = 8.5, 2 H, *H* ortho to O); 6.82 (d, J = 9.0, 1 H, N*H*CH); 5.73 (app ddt, 1 H, J = 17.0, J = 10.5, J = 7.0, H_2CCH=CH_2); 5.04 (s, 2 H, OCH_2Ph); 4.96-5.02 (m, 2 H, CH=CH_2); 4.02 (app dt, J = 4.5, J = 9.0, 1 H, NHC*H*); 3.00-3.20 (m, 2 H, NHCH_2); 2.79 (dd, J = 9.0, J = 13.5, 1 H, CHC*H*^{a}H^{b}Ar); 2.64 (dd, J = 9.0, J = 13.5, 1 H CHCH^a*H*^{b}Ar); 2.10 (app q, J = 7.0, 2 H, CH_2CH_2CH=CH_2); 1.29 (s, 9 H, C(CH_3)$_3$). ^{13}C NMR (DMSO d_6): 171.4 (C=O); 156.8 (C=O); 155.1 (C); 137.2 (C); 135.9 (CH); 130.2 (C); 130.1 (CH); 128.3 (CH); 127.7 (CH); 127.5 (CH); 116.1 (CH_2); 114.3 (CH); 77.9 (C); 69.0 (CH_2); 55.9 (CH); 37.9 (CH_2); 36.8 (CH_2); 33.3 (CH_2); 28.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 2977; 2934; 1711 (C=O stretch); 1676 (C=O stretch); 1611; 1506.

HRMS calc. for $C_{25}H_{32}N_2O_4$: 425.2440 $[M+H]^+$; found 425.2448.

$(\alpha)^{16}_D$ +14.7 (*c* 0.63, $CHCl_3$)

28 – 11-Oxa-2,4,9-triazatridec-3-enoic acid, 8-[(3-buten-1-ylamino)carbonyl]-12,12-dimethyl-10-oxo-3-[[(phenylmethoxy)carbonyl]amino]-, phenylmethyl ester, (8S)



BocArg(diZ)OH (869 mg, 1.6 mmol) *C*-functionalized with homoallyl amine according to protocol I yielding **28** as a waxy solid (520 mg, 54%).

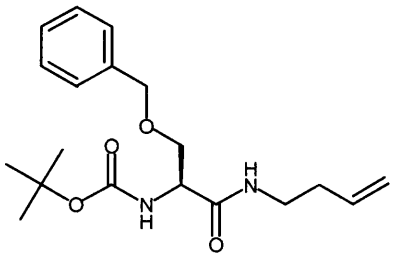
^{1}H NMR (DMSO d_6): 9.18 (br s, (HN)$_2$C=N); 7.78 (app t, J = 5.5, HNCH$_2$); 7.26-7.43 (m, 10 H, Ar-H); 6.77 (d, J = 8.1, 1 H, NHCH); 5.71 (app ddt, J = 17.0, J = 10.0, J = 6.5, 1 H, H$_2$CCH=CH$_2$); 5.23 (s, 2 H, CH_2Ph); 5.05 (s, 2 H, CH_2Ph); 4.99 (d, J = 17.0, 1 H, CH=CH_{trans}); 4.95 (d, J = 10.0, 1 H, CH=CH_{cis}); 3.85 (br m, 3 H, NHCHCH$_2$CH$_2$CH_2NH); 2.95-3.20 (m, 2 H, CONHCH_2); 2.10 (app q, J = 6.5, 2 H, CH_2CH=CH$_2$); 1.4-1.7 (m, 4 H, NHCHCH_2CH_2CH$_2$N); 1.35 (s, 9 H, C(CH_3)$_3$). ^{13}C NMR (DMSO d_6): 171.6 (C=O); 162.8 (C=O); 159.5 (app t, C=N); 155.1 (C=O); 154.9 (C=O); 137.0 (C); 135.8 (CH); 135.2 (CH); 128.4 (CH); 128.2 (CH); 128.19 (CH); 127.8 (CH); 127.78 (CH); 127.6 (CH); 116.1 (CH$_2$); 77.9 (C); 68.1 (CH$_2$); 66.0 (CH$_2$); 54.0 (CH); 44.3 (CH$_2$); 37.8 (CH$_2$); 33.3 (CH$_2$); 29.3 (CH$_2$); 28.1 (CH$_3$); 25.1 (CH$_2$).

IR, solution cell, DCM (cm^{-1}): 3665 (N-H stretch); 3391 (N-H stretch); 3281 (N-H stretch); 3038; 2974; 2941; 1715 (C=O stretch); 1668 (C=O stretch); 1612; 1506.

HRMS calc. for $C_{31}H_{41}N_5O_7$: 596.3079 [M+H]$^+$; found 596.3085.

$(\alpha)^{25}_D$ +15.1 (*c* 0.29, $CHCl_3$).

29 – ((S)-2-Benzyloxy-1-but-3-enylcarbamoyl-ethyl)-carbamic acid tert-butyl ester



BocSer(Bzl)OH (1.0 g, 3.4 mmol) was *C*-functionalized with homoallyl amine according to protocol I. Purification gave **29** as a colourless liquid (541 mg, 46%).

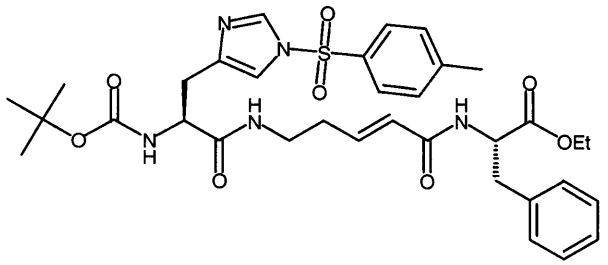
^{1}H NMR ($CDCl_3$): 7.26-7.40 (m, 5 H, Ar-*H*); 6.49 (br t, *J* = 5.5, 1 H, N*H*CH_2); 5.71 (ddt, *J* = 17.0, *J* = 10.0, *J* = 6.5, 1 H, H_2CC*H*=CH_2); 5.41 (br, 1 H, N*H*); 4.92-5.00 (m, 2 H, H_2CCH=C*H*$_2$); 4.48 (d, *J* = 12.0, 1 H, OC*H*^{a}H^{b}Ph); 4.42 (d, *J* = 12.0, 1 H, OCH^a*H*^{b}Ph); 4.19 (br, 1 H, NHC*H*); 3.81 (dd, *J* = 4.0, *J* = 9.0, 1 H, NHCHC*H*^{a}H^b); 3.50 (dd, *J* = 9.0, *J* = 6.5, 1 H, NHCHCH^a*H*^{b}); 3.17-3.34 (m, 2 H, NHC*H*$_2$); 2.16 (app q, *J* = 6.5, 2 H, CH_2C*H*$_2$CHC*H*$_2$); 1.39 (s, 9 H, C(C*H*$_3$)$_3$). ^{13}C NMR (MeOH d_4): 172.8 (C=O); 157.6 (C=O); 139.2 (C); 136.6 (CH); 129.4 (CH); 128.9 (CH); 128.8 (CH); 117.1 (CH_2); 80.9 (C); 74.1 (CH_2); 71.1 (CH_2); 56.2 (CH); 39.9 (CH_2); 34.6 (CH_2); 28.7 (CH_3).

HRMS calc. for $C_{22}H_{30}N_4O_5S$: 371.1941 $[M+Na]^+$; found 371.1930.

IR, solution cell, DCM (cm^{-1}): 3425 (N-H stretch); 2978; 2872; 1713 (C=O stretch); 1674 (C=O stretch).

$(\alpha)^{19}_D$ +9.4 (*c* 0.64, $CHCl_3$).

30 – (S)-2-((E)-5-{(S)-2-*tert*-Butoxycarbonylamino-3-[1-(toluene-4-sulfonyl)-1H-imidazol-4-yl]-propionylamino}-pent-2-enoylamino)-3-phenyl-propionic acid ethyl ester



Method 1: **26** (200 mg, 0.43 mmol), **19** (100 mg, 0.40 mmol) and **GII** (33 mg, 10 mol%) were reacted according to protocol III. After 48 h **26** and **19** were still visible by TLC, however the reaction was terminated. **30** was obtained as a red solid (45 mg, 16%). Starting materials **26** (124 mg) and **19** (59 mg) were recovered, exhibiting red colouration.

Method 2: **26** (125 mg, 0.27 mmol), **19** (56 mg, 0.23 mmol) and **GII** (18 mg, 9 mol%) were reacted according to protocol III. After 6 days **26** and **19** were still visible by TLC, however the reaction was terminated. **30** was obtained as a red solid (45 mg, 29%).

Method 3: **26** (116 mg, 0.25 mmol), **19** (50 mg, 0.20 mmol) and **GII** (28 mg, 17 mol%) were reacted according to protocol III, except chloroform was used in place of DCM. After 6 days **26** and **19** were still visible by TLC, however the reaction was terminated. **30** was obtained as a red solid (49 mg, 36%).

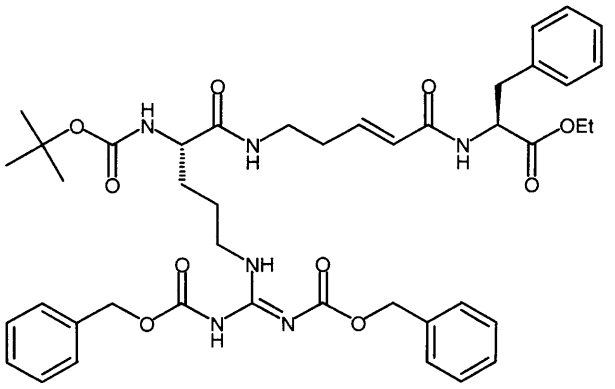
Method 4: A solution of **26** (58 mg, 0.13 mmol), **19** (25 mg, 0.10 mmol) and **GII** (16 mg, 19 mol%), in DCM (1.4 mL) and DMF (3 drops) was sealed in a microwave vial, the solution was subjected to microwave irradiation (150 W, 90°C) for 15 min. After cooling, the mixture was irradiated for a further 15 min and allowed to cool to RT. The solvent was removed *in vacuo*, a small amount of DCM added to the resultant material and this was purified using silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate), giving **30** as a red waxy solid (28 mg, 41%).

^{1}H NMR (DMSO d_6, 353 K, 400 MHz): 8.02 (d, J = 7.5, 1 H, N*H*); 7.75 (app t, J = 5.8, 1 H, N*H*); 7.27 (d, J = 8.0, 2 H, Ts-*H*); 7.19-7.27 (m, 5 H, Phe-H); 7.09 (d, J = 8.0, 5 H, Ts-*H*); 6.65 (br, 1 H, N*H*); 6.57 (app dt, J = 15.5, J = 7.0, 1 H, CH_2CH=CH); 6.00 (d, J = 15.5, 1 H, CH_2CH=C*H*); 4.57 (app dt, J = 6.5, J = 8.0, 1 H, NHC*H*CH_2Ph); 4.24 (app dt, J = 5.5, J = 8.5, 1 H, NHC*H*CH_2Im); 4.05 (app q, J = 7.0, 2 H, OCH_2CH_3); 3.10-3.22 (m, 2 H, CONHCH_2); 3.10 (dd, J = 15.0, J = 5.5, 1 H, C*H*^{a}H^{b}Im); 3.05 (dd, J = 14.0, J = 6.5, 1 H, C*H*^{a}H^{b}Ph); 2.95 (dd, J = 14.0, J = 8.5, 1 H, CH^a*H*^{b}Ph); 2.90 (dd, J = 15.0, J = 8.5, 1 H, CH^a*H*^{b}Im); 2.29 (s, 3 H, ArCH_3); 2.23 (app q, J = 6.5, 2 H, NHCH$_2$CH_2); 1.34 (s, 9 H, C(CH_3)$_3$); 1.12 (app t, J = 7.0, 3 H, OCH$_2$CH_3). ^{13}C NMR ($CDCl_3$): 171.9 (C=O); 171.2 (C=O); 165.1 (C=O); 155.6 (C=O); 146.5; 141.4; 140.5; 136.4; 136.1; 134.7; 130.5 (CH); 129.3 (CH); 128.5 (CH); 127.5 (CH); 127.0 (CH); 125.2 (CH); 114.9; 80.2 (C); 61.5 (CH_2); 54.1 (CH); 53.3 (CH); 38.0 (CH_2); 37.9 (CH_2); 32.1 (CH_2); 30.3 (CH_2); 28.3 (CH_3); 21.7 (CH_3); 14.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3422 (N-H stretch); 3296 (N-H stretch); 2932; 1762 (C=O stretch); 1674 (C=O stretch); 1501.

HRMS calc. for $C_{34}H_{43}N_5O_8S$: 682.2905 $[M+H]^+$; found 682.2920.

31 – 2,4,10,16-Tetraazaoctadeca-3,13-dienedioic acid, 8-[[(1,1-dimethylethoxy)carbonyl]amino]-9,15-dioxo-3-[[(phenylmethoxy)carbonyl]amino]-17-(phenylmethyl)-, 18-ethyl 1-(phenylmethyl) ester, (8S,13E,17S)



Method 1: **28** (134 mg, 0.23 mmol), **19** (52 mg, 0.21 mmol) and **GII** (21 mg, 12 mol%) were reacted according to protocol III affording **31** as a red waxy solid (120 mg, 70%).

Method 2: **28** (66 mg, 0.11 mmol) and **19** (25 mg, 0.10 mmol) were reacted according to protocol IV yielding **31** as a red waxy solid (32 mg, 40%). Repetition afforded 43%.

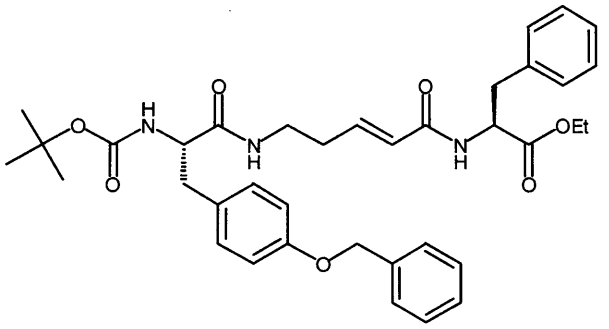
Method 3: **28** (89 mg, 0.15 mmol) and **19** (25 mg, 0.10 mmol) were reacted according to protocol IV yielding **31** as a red waxy solid (45 mg, 55%).

^{1}H NMR (400 MHz, 353 K) (DMSO d_6): 8.98 (s, 2 H, (HN)$_2$C=N); 7.93 (br d, 1 H, NHCHCH$_2$Ph); 7.56 (br t, 1 H, NHCH_2); 7.19-7.40 (m, 15 H, Ar-H); 6.57 (app dt, 1 H, J = 15.5, J = 7.0, CH$_2$CH=CH); 6.23 (br m, 1 H, NHCHCH$_2$CH$_2$); 5.98 (d, J = 15.5, 1 H, CH$_2$CH=CH); 5.25 (app s, 2 H, OCH_2Ph); 5.07 (app s, 2 H, OCH_2Ph); 4.58 (ddd, J = 8.5, J = 8.0, J = 6.0, 1 H, NHCHCH$_2$Ph); 4.05 (app q, J = 7.0, 2 H, OCH_2); 3.87 (m, 3 H, NHCHCH$_2$CH$_2$CH_2N=C); 3.12 (m, 2 H, CONCH_2); 3.05 (dd, J = 6.0, J = 13.5, 1 H, CH^aH^{b}Ph); 2.98 (dd, J = 8.5, J = 13.5, 1 H, CH^aH^bPh); 2.23 (app q, 2 H, J = 7.0, H_2CCH=C); 1.45-1.65 (m, 4 H, NHCHCH_2CH_2CH$_2$N=C); 1.36 (s, 9 H, C(CH_3)$_3$); 1.12 (app t, J = 7.0, 3 H, OCH$_2$CH_3). ^{13}C NMR ($CDCl_3$): 172.0 (C=O); 171.7 (C=O); 165.1 (C=O); 163.4 (C=O); 160.9 (C=N); 155.8 (C=O); 155.5 (C=O); 141.3 (CH); 136.4 (C); 136.0 (C); 134.6 (C); 129.3 (CH); 128.8 (CH); 128.6 (CH); 128.5 (CH); 128.3 (CH); 128.2 (CH); 127.0 (CH); 124.9 (CH); 79.6 (C); 69.0 (CH_2); 67.2 (CH_2); 61.5 (CH_2); 53.5 (CH); 53.2 (CH); 44.1 (CH_2); 37.9 (CH_2); 37.7 (CH_2); 31.9 (CH_2); 29.0 (CH_2); 28.4 (CH_3); 24.5 (CH_2); 14.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3391 (N-H stretch); 2976; 2939; 1719 (C=O stretch); 1676 (C=O stretch); 1611; 1504.

HRMS calc. for $C_{43}H_{54}N_6O_{10}$: 815.3974 $[M+H]^+$; found 815.3979.

32 – (S)-2-{(E)-5-[(S)-3-(4-Benzyloxy-phenyl)-2-*tert*-butoxycarbonylamino-propionylamino]-pent-2-enoylamino}-3-phenyl-propionic acid ethyl ester



Method 1: **27** (95 mg, 0.23 mmol), **19** (52 mg, 0.21 mmol) and **GII** (19 mg, 11 mol%) were reacted according to protocol III yielding a **32** as a pale brown waxy solid (120 mg, 70%).

Method 2: A solution of **27** (53 mg, 1.3 mmol), **19** (25 mg, 0.10 mmol) and **GII** (16 mg, 19 mol%) in DCM (1.4 mL) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 90°C) for 15 min. The mixture was irradiated for a further three periods of 15 min, after which time **27** was no longer visible by TLC. The reaction mixture was allowed to cool to RT, and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) affording **32** as a pale brown waxy solid (37 mg, 57%).

Optimization: A solution of **27** and **19** in DCM (0.5 mL) was added to a microwave vial. Molar ratios are shown in Table 1. The smallest molarity used was 0.1 mmol in every case. A Grubbs catalyst was added, the vial sealed, and the mixture subjected to microwave irradiation for 30 mins. The reaction mixture was then degassed with argon for approximately 30 seconds, and the mixture subjected to microwave irradiation for a further 30 mins. The reaction mixture was allowed to cool to RT, and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and

purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate). All conditions and yields are reported in Table 1.

Table 1

Mol(**27**)/ Mol(**19**)	Mol% **GII**	Catalyst	Temp (°C)	Power (W)	Yield (%)
1.3	18	**GII**	80	150	55
1.3	18	**GII**	90	300	66
0.83	26	**GII**	90	300	61
1.3	18	**HG**	90	300	36
2.1	20	**GII**	90	300	52
0.83	18 §	**GII**	90	300	58
1.3	18	**GII**	100	300	82
1.3	18	**GII**	110	300	59
1.3	18	**GII**	100	*n/a**	58
1.3	18	**GII**	95	*n/a**	77

§ Catalyst added in 2 x 9 μmol portions, one initially, the second after 30 min

* Used a Biotage Initiator 60 microwave unit

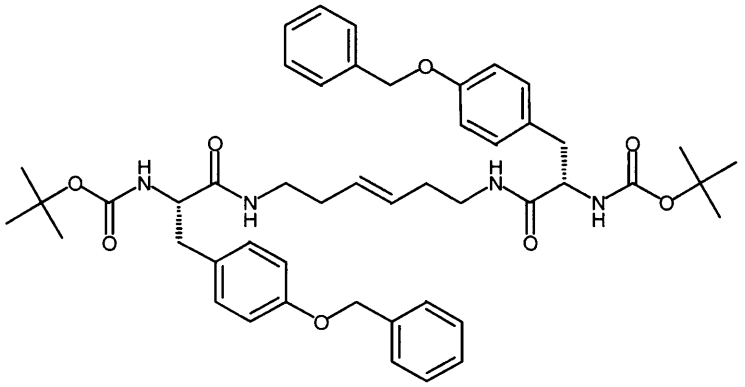
^{1}H NMR (MeOH d_4, 400 MHz, 333 K): 7.16-7.39 (m, 10 H, Ar-*H*); 7.10 (d, *J* = 8.5, 2 H, *H* meta to O); 6.89 (d, *J* = 8.5, 2 H, *H* ortho to O); 6.64 (app dt, *J* = 15.5, *J* = 7.0, 1 H, CH_2CH=CH); 5.94 (d, *J* = 15.5, 1 H, CH_2CH=C*H*); 5.03 (s, 2 H, OCH_2Ph); 4.71 (dd, 1 H, *J* = 6.5, *J* = 8.0, NHC*H*CH_2Ar); 4.18 (app t, *J* = 7.0, BocNHC*H*CH_2Ar); 4.10 (app q, *J* = 7.0, 2 H, OCH_2CH_3); 3.15-3.24 (m, 2 H, CONHCH_2); 3.11 (dd, *J* = 6.5, *J* = 14.0, 1 H, C*H*^{a}H^{b}Ph); 2.99 (dd, *J* = 8.0, *J* = 14.0, 1 H, CH^a*H*^{b}Ph); 2.95 (dd, *J* = 6.5, *J* = 13.8, 1 H, C*H*^{a}H^{b}Ar); 2.67 (dd, *J* = 8.0, *J* = 13.8, 1 H, CH^a*H*^{b}Ar); 2.27 (app q, *J* = 7.0, 2 H, H_2CCH=C); 1.35 (s, 9 H, C(CH_3)$_3$); 1.18 (app t, *J* = 7.0, 3 H, OCH_2CH_3). ^{13}C NMR ($CDCl_3$): 172.0 (C=O); 171.7 (C=O); 165.1 (C=O); 157.7 (C=O); 155.6 (C); 141.1 (CH); 137.0 (C); 136.1 (C); 130.4 (C); 130.4 (CH); 129.3 (CH); 128.6 (CH); 128.5 (CH); 128.0 (CH); 127.5 (CH); 127.0 (CH); 125.0 (CH); 115.0 (CH_2); 80.0 (C); 70.1 (CH_2); 61.5

(CH_2); 53.4 (CH_2); 56.0 (CH); 53.4 (CH); 38.2 (CH_2); 38.0 (CH_2); 37.6 (CH_2); 32.0 (CH_2); 28.3 (CH_3); 14.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3425 (N-H stretch); 2979; 2933; 1726 (C=O stretch); 1678 (C=O stretch); 1506.

HRMS calc. for $C_{37}H_{45}N_3O_7$: 666.3150 $[M+Na]^+$; found 666.3142.

32hd – ((S)-2-(4-Benzyloxy-phenyl)-1-{6-[(S)-3-(4-benzyloxy-phenyl)-2-tert-butoxycarbonylamino-propionylamino]-hex-3-enylcarbamoyl}-ethyl)-carbamic acid tert-butyl ester (*Homodimer*)

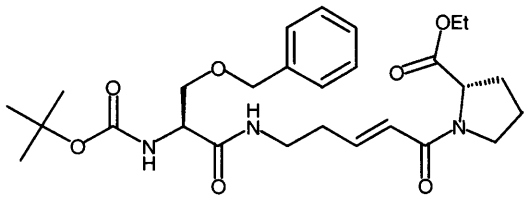


^1H NMR ($CDCl_3$): 7.23-7.60 (m, 10 H, Ar-H); 7.10 (d, *J* = 8.5, 4 H, *H* ortho to O); 6.89 (d, *J* = 8.5, 4 H, *H* meta to O); 6.75 (br, 2 H, N*H*CH_2); 5.51 (d, J = 8.5, 2 H, N*H*CH); 5.26 (br m, 2 H, C*H*=C*H*); 4.99 (s, 4 H, OCH_2Ph); 4.38 (br m, 2 H, NHC*H*); 3.50 (br m, 2 H, NHC*H*^{a}H^bCH_2); 2.97 (dd, *J* = 7.0, *J* = 14.0, 2 H, C*H*^{a}H^{b}Ar); 2.85 (dd, *J* = 7.5, *J* = 14.0, 2 H, CH^a*H*^{b}Ar); 2.81-3.16 (obsc, 2 H, NHCH^a*H*^{b}CH_2); 2.13 (br m, 2 H, C*H*^{a}H^{b}CH=CH); 2.04 (br, 2 H, CH^a*H*^{b}CH=CH); 1.36 (s, 6 H, COCH_3). ^{13}C NMR ($CDCl_3$): 172.1 (C=O); 157.6 (C=O); 155.8 (C); 137.0 (C); 130.3 (CH); 129.8 (CH); 129.5 (CH); 128.6 (CH); 127.9 (CH); 127.4 (CH); 114.8 (CH); 79.8 (C); 70.0 (CH_2); 56.3 (CH); 38.6 (CH_2); 38.1 (CH_2); 32.6 (CH_2); 28.3 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 3352; 2932; 1668 (C=O stretch); 1508.

HRMS calc. for $C_{48}H_{60}N_4O_8$: 843.4303 $[M+Na]^+$; found 843.4320.

33 – (S)-1-[(E)-5-((S)-3-Benzyloxy-2-*tert*-butoxycarbonylamino-propionylamino)-pent-2-enoyl]-pyrrolidine-2-carboxylic acid methyl ester



Method 1: **29** (104 mg, 0.30 mmol) and **23** (42 mg, 0.23 mmol) were reacted according to protocol III, using chloroform in place of DCM. Purification yielded **33** as a brown waxy solid (56 mg, 49%)

Method 2: A solution of **29** (88 mg, 1.3 mmol) and **23** (25 mg, 0.10 mmol) and **GII** (16 mg, 10 mol%) in DCM (1.4 mL) was sealed in a microwave vial. The solution was subjected to microwave irradiation (150 W, 60°C) for 2 h. Purification of the product mixture using silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate) yielded **33** as a brown waxy solid (35 mg, 35%).

Method 3: **29** (35 mg, 0.12 mmol) and **23** (19 mg, 0.10 mmol) were reacted according to protocol IV. Purification yielded **33** as a brown waxy solid (25 mg, 50%).

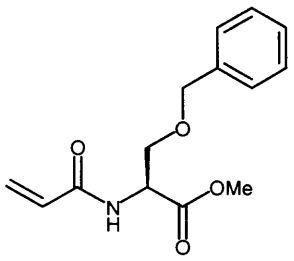
^{1}H NMR ($CDCl_3$, 400 MHz): 7.23-7.33 (m, 5 H, Ph-*H*); 6.81 (app dt, *J* = 14.0, *J* = 6.5, 1 H, CH_2CH=CH); 6.51 (br, 1 H, N*H*); 6.15 (d, *J* = 15.2, 1 H, CH_2CH=C*H*); 5.29 (d, *J* = 5.5, 1 H, N*H*CH); 4.51 (AB q, *J* = 11.8, 2 H, OCH_2Ph); 4.41-4.52 (br, 1 H, $CHCO_2Me$); 4.20 (app dt, *J* = 4.5, *J* = 6.5, 1 H, $NHCHCH_2O$); 3.84 (dd, *J* = 9.5, *J* = 4.5, 1 H, $CHCH^aH^bO$); 3.68 (s, 3 H, OMe); 3.64 (br t, 1 H, $NCH^aH^bCH_2CH_2$); 3.56 (dd, *J* = 6.5, *J* = 9.5, 1 H, $CHCH^aH^bO$); 3.51-3.57 (obsc, 1 H, $NCH^aH^bCH_2CH_2$); 3.32-3.42 (m, 2 H, $NHCH_2$); 2.37 (app q, *J* = 6.5, 2 H, $NHCH_2CH_2$); 1.86-2.20 (m, 4 H, $NCH_2CH_2CH_2$); 1.41 (s, 9 H, $C(CH_3)_3$). ^{13}C NMR ($CDCl_3$): 172.7 (C=O); 170.3 (C=O); 164.4 (C=O); 155.5 (C=O); 142.6 (CH); 137.5 (C); 128.5 (CH); 127.9 (CH); 127.7 (CH); 123.0 (CH);

80.2 (C); 73.4 (CH_2); 69.9 (CH_2); 59.2[§]; 58.9 (CH_3); 52.6[§]; 54.0 (CH); 52.2 (CH); 46.8 (CH_2); 46.5[§]; 38.2 (CH_2); 32.3 (CH_2); 31.3[§]; 29.1 (CH_2); 28.3 (CH_3); 24.8 (CH_2); 22.5[§].

IR, solution cell, DCM (cm^{-1}): 3886; 3425 (N-H stretch); 2978; 2949; 2878; 1715 (C=O stretch); 1672 (C=O stretch); 1616.

HRMS calc. for $C_{26}H_{36}N_3O_7$: 504.2710 $[M+H]^+$; found 504.2714.

34 – (S)-2-Acryloylamino-3-benzyloxy-propionic acid methyl ester



To a stirred solution of BocSer(Bzl)OH (1.8 g, 6.1 mmol) in methanol (10 mL) and toluene (10 mL) was added 2 M TMS diazomethane in hexanes (3.1 mL, 6.2 mmol). Acetic acid was added dropwise until the yellow colour disappeared. The solvent was removed *in vacuo*, and a further 20 mL of toluene added. This was removed *in vacuo* in order to azeotrope off impurities. TFA (5 mL) and DCM (12 mL) were added and the mixture monitored by LCMS until the Boc protected material could no longer be seen. Toluene (10 mL) was added and the solvents removed *in vacuo*. This was repeated once. The resultant material was dissolved in DCM (40 mL) and triethylamine (0.12 mL, 0.76 mmol) added. Acryloyl chloride in DCM (0.17 mmol per mL) was slowly added until the starting free amine was no longer visible by LCMS. The solvent was removed *in vacuo* and the material worked up as described in protocol II, yielding **34** as a colourless viscous liquid (1.24 g, 77%).

^{1}H NMR ($CDCl_3$, 400 MHz): 7.25-7.36 (m, 5 H, Ph-*H*); 6.47 (d, *J* = 8.0, 1 H, N*H*); 6.34 (dd, *J* = 1.5, *J* = 17.0, 1 H, *trans* *H*HC=CH); 6.16 (dd, *J* = 10.0, *J* = 17.0, 1 H, H_2C=C*H*);

[§] Smaller peaks corresponding to a different conformation of the proline portion of the compound. Analogous to those seen in **22**.

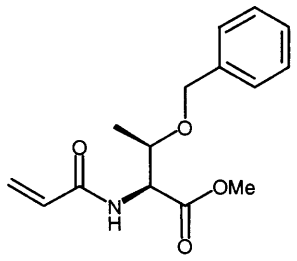
5.69 (dd, J = 1.5, J = 10.0, 1 H, *cis* H*H*C=CH); 4.84 (app dt, J = 3.0, J = 8.0, 1 H, NHC*H*); 4.51 (AB q, J = 12.0, 2 H, CH_2OCH_2Ph); 3.93 (dd, J = 3.0, J = 9.5, 1 H, CHC*H*^{a}H^{b}O); 3.94 (s, 3 H, OMe); 3.93 (dd, J = 3.0, J = 9.5, 1 H, CHCH^a*H*^{b}O). ^{13}C NMR ($CDCl_3$, 100 MHz): 171.1 (C=O); 165.6 (C=O); 137.9 (C); 130.7 (CH); 128.9 (CH); 128.3 (CH); 128.0 (CH); 127.7 (CH_2); 73.7 (CH_2); 70.1 (CH_2); 53.1 (CH); 53.0 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 3071; 3034; 2951; 2872; 1747 (C=O stretch); 1678 (C=O stretch); 1626; 1506.

HRMS calc. for $C_{14}H_{17}NO_4$: 264.12360 $[M+H]^+$; found 264.12230.

$(\alpha)^{25}_D$ +47.2 (*c* 0.46 $CHCl_3$)

35 – (2S,3R)-2-Acryloylamino-3-benzyloxy-butyric acid methyl ester



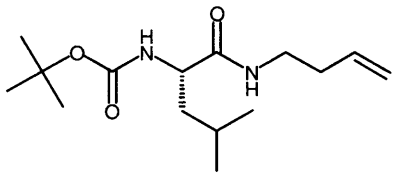
BocThr(Bzl)OH (472 mg, 1.5 mmol) was treated with a series of reagents, as described for Compound **34**. **35** was obtained as a colourless viscous liquid (414 mg, 99%).

^{1}H NMR ($CDCl_3$, 400 MHz): 7.20-7.32 (m, 5 H, Ph-*H*); 6.30 (dd, J = 1.5, J = 17.0, 1 H, *H*^{a}H^{b}C=CH); 6.25 (br s, 1 H, N*H*); 6.16 (dd, J = 10.0, J = 17.0, 1 H, H_2C=C*H*); 5.66 (dd, J = 1.5, J = 10.0, 1 H, H^a*H*^{b}C=CH); 4.66 (dd, J = 2.5, J = 9.5, 1 H, NHC*H*); 4.54 (d, J = 12.0, 1 H, C*H*^{a}H^{b}Ph); 4.34 (d, J = 12.0, 1 H, CH^a*H*^{b}Ph); 4.13 (dq, J = 2.5, J = 6.5, 1 H, C*H*(CH_3)OCH_2); 3.63 (s, 3 H, OMe); 1.19 (d, J = 6.5, 3 H, C*H*$_3$CHOCH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): 171.4 (C=O); 166.1 (C=O); 138.1 (C); 130.7 (CH); 128.8 (CH); 128.3 (CH); 128.2 (CH); 127.8 (CH_2); 74.8 (CH); 71.3 (CH_2); 57.0 (CH_3); 52.7 (CH_3); 16.7 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 3069; 3034; 2984; 2947; 2874; 1746 (C=O stretch); 1678 (C=O stretch); 1628; 1508.

HRMS calc. for $C_{15}H_{19}NO_4$: 278.1392 $[M+H]^+$; found 278.13840.
$(\alpha)^{25}_D$ +13.0 (*c* 0.70, $CHCl_3$)

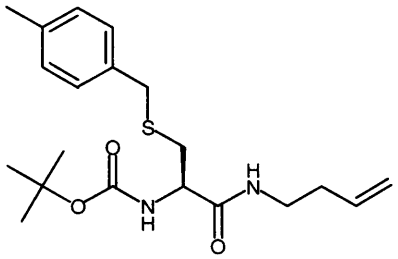
36 – (S)-(1-but-3-enylcarbamoyl-3-methyl-butyl)-carbamic acid tert-butyl ester[93]



To as stirred solution of BocLeuOH (1.0 g, 4.0 mmol) in DMF (5 mL) and DCM (10 mL) was added HATU (1.5 g, 3.9 mmol) and DIPEA (2 mL, 11.5 mmol) and the resultant mixture stirred for 10 minutes. A solution of homoallyl amine hydrochloride (395 mg, 3.66 mmol) and DIPEA (0.5 mL, 2.9 mmol) in DCM (2 mL) was added and the mixture monitored by LCMS. After 2 h successive LCMS readings showed the relative intensities of the peaks to be constant. The solvent was removed *in vacuo*, DCM (15 mL) was added and worked up as described in Protocol I yielding **36** as a white waxy solid (940 mg, 89%).

^{1}H NMR (MeOH d_4, 400 MHz): 5.81 (app ddt, J = 17.0, J = 10.5, J = 7.0, 1 H, H_2CCH=CH_2); 5.10 (app dd, J = 17.0, J = 1.5, 1 H, *H*HC=CH); 5.05 (d, J = 10.5, 1 H, H*H*C=CH); 4.05 (dd, J = 9.0, J = 6.0, 1 H, NHC*H*); 3.15: 3.33 (m, 2 H, NHCH_2); 2.26 (app q, J = 7.0, 2 H, $NHCH_2CH_2$); 1.61-1.72 (m, 1 H, C*H*$(CH_3)_2$); 1.46-1.52 (m, 2 H, $CHCH_2CH$); 1.52 (s, 9 H, $C(CH_3)_3$); 0.93-0.97 (m, 6 H, $CH(CH_3)_2$). ^{13}C NMR (MeOH d_4, 100 MHz): 176.1 (C=O); 137.0 (CH); 117.5 (CH_2); 81.0 (C); 55.1 (CH); 42.9 (CH_2); 40.2 (CH_2); 35.1 (CH_2); 29.1 (CH_3); 26.3 (CH); 23.8 (CH_3); 22.4 (CH_3).
IR, solution cell, DCM (cm^{-1}): 3431 (N-H stretch); 3342 (N-H stretch); 3055; 2966; 2876; 1707 (C=O stretch); 1676 (C=O stretch); 1501.
HRMS calc. for $C_{15}H_{28}N_2O_3$: 285.2178 $[M+H]^+$; found 285.21730.
$(\alpha)^{25}_D$ -39.2 (*c* 0.47, $CHCl_3$).

37 – [(R)-1-But-3-enylcarbamoyl-2-(4-methyl-benzylsulfanyl)-ethyl]-carbamic acid tert-butyl ester



To a stirred solution of BocCys(4-MeBzl)OH (2.0 g, 6.2 mmol) in DCM (5 mL) and DMF (5 mL) was added HOBt (873 mg, 6.5 mmol), HBTU (2.3 g, 6.1 mmol) and DIPEA (1.1 mL, 6.2 mmol), and the resultant mixture stirred for 20 min. A solution of homoallyl amine hydrochloride (335 mg, 3.1 mmol) and DIPEA (0.6 mL, 3.4 mmol) in DCM (2 mL) was added, and the resultant solution stirred for 3 h. The majority of the solvent was removed *in vacuo*. DCM (20 mL) was then added, and the mixture washed with HCl (2x10 mL, 1 M), LiCl (2x10ml, 10%), $NaHCO_3$ (2x10 mL, 0.5 M), water (2x10 mL) and saturated brine (10 mL) and dried with anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration and the solvent removed *in vacuo*. The resultant material was dissolved in a small amount of DCM and purified by silica-gel chromatography (petroleum ether and ethyl acetate, initial 3:1 v:v, final 100% ethyl acetate), affording **37** as a waxy solid (989 mg, 84%).

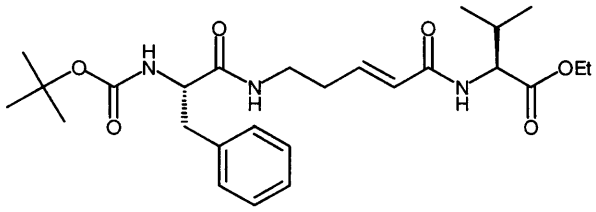
^{1}H NMR ($CDCl_3$): 7.22 (d, J = 8.0, 2 H, Ph-*H*); 7.12 (d, J = 8.0, 2 H, Ph-*H*); 6.26 (br t, 1 H, N*H*CH_2); 5.75 (app ddt, J = 17.0, J = 10.5, J = 7.0, 1 H, H_2CC*H*=CH_2); 5.26 (br m, 1 H, N*H*CH); 5.06-5.13 (m, 2 H, H_2C=CH); 4.18 (br app q, J = 6.0, 1 H, NHC*H*); 3.71 (s, 2 H, SCH_2Ar); 3.29-3.36 (m, 2 H, NHCH_2); 2.86 (dd, J = 5.5, J = 14.0, 1 H, CHC*H*^{a}H^{b}S); 2.70 (dd, J = 9.5, J = 14.0, 1 H, CHCH^a*H*^{b}S); 2.32 (s, 3 H, ArCH_3); 2.22-2.29 (m, 2 H, NHCH$_2$CH_2); 1.45 (s, 9 H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$): 170.5 (C=O); 155.4 (C=O); 136.9 (C); 135.0 (CH); 134.9 (C); 129.3 (CH); 128.9 (CH); 117.4 (CH_2); 80.3 (C); 53.8 (CH); 38.6 (CH_2); 36.2 (CH_2); 33.8 (CH_2); 33.6 (CH_2); 28.3 (CH_3); 21.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3423 (N-H stretch); 2974; 2930; 2330; 1713 (C=O stretch); 1676 (C=O stretch); 1489.

HRMS calc. for $C_{20}H_{30}N_2O_3S$: 379.2055 $[M+H]^+$; found 379.20450.

$(\alpha)^{19}_D$ +3.9 (*c* 0.42, $CHCl_3$)

38 – (S)-2-[(E)-5-((S)-2-*tert*-Butoxycarbonylamino-3-phenyl-propionylamino)-pent-2-enoylamino]-3-methyl-butyric acid ethyl ester



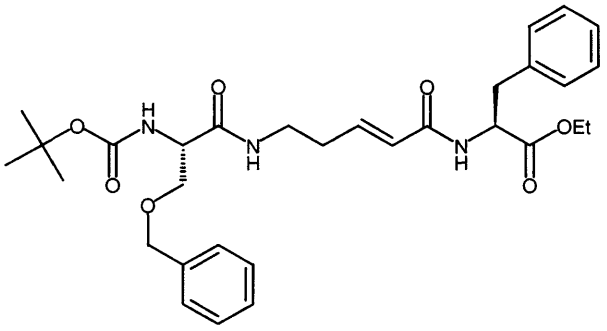
11 (70 mg, 0.22 mmol) and **22** (40 mg, 0.20 mmol) were reacted according to protocol IV yielding **38** as a brown waxy solid (33 mg, 34%). Repetition with **11** (35 mg, 0.11 mmol) and **22** (20 mg, 0.0.10 mmol) in the Biotage Initiator 60 microwave unit afforded 14.9 mg (30%) material.

^1H NMR ($CDCl_3$, 400 MHz): 7.22-7.32 (m, 5 H, Ph-*H*); 6.68 (dt, *J* = 15.0, *J* = 6.5, 1 H, CH_2CH=CH); 6.52 (br d, 1 H, N*H*(Phe)); 6.29 (br t, 1 H, N*H*CH_2); 5.93 (d, *J* = 15.0, 1 H, CH_2CH=C*H*); 4.65 (dd, *J* = 5.0, *J* = 9.0, 1 H, NHC*H*(Val)); 4.42 (br m, 1 H, NHC*H*(Phe)); 4.18-4.42 (m, 2 H, OCH_2CH_3); 3.20-3.40 (m, 2 H, NHCH_2); 3.04 (d, *J* = 7.0, 2 H, CHCH_2); 2.15-2.35 (m, 3 H, $NHCH_2CH_2$ and C*H*$(CH_3)_2$ superimposed); 1.40 (s, 9 H, C$(CH_3)_3$); 1.32 (app t, *J* = 7.0, OCH_2CH_3); 0.98 (app t, *J* = 7.0, $CH(CH_3)_2$). ^{13}C NMR (MeOH d_4, 100 MHz): 174.8 (C=O); 173.5 (C=O); 168.8 (C=O); 158.0 (C=O); 143.26 (CH); 139.0 (C); 130.8 (CH); 129.8 (CH); 128.1 (CH); 126.4 (CH); 81.0 (C); 62.6 (CH_2); 59.7 (CH); 58.0 (CH); 40.0 (CH_2); 39.5 (CH_2); 33.2 (CH_2); 32.3 (CH); 29.1 (CH_3); 19.9 (CH_3); 19.0 (CH_3); 15.0 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3427 (N-H stretch); 3329 (N-H stretch); 3057; 2974; 2936; 1726 (C=O stretch); 1678 (C=O stretch); 1504.

HRMS calc. for $C_{26}H_{39}N_3O_6$: 490.2917 $[M+H]^+$; found 490.2925.

39 – (S)-2-[(E)-5-((S)-3-Benzyloxy-2-*tert*-butoxycarbonylamino-propionylamino)-pent-2-enoylamino]-3-phenyl-propionic acid ethyl ester



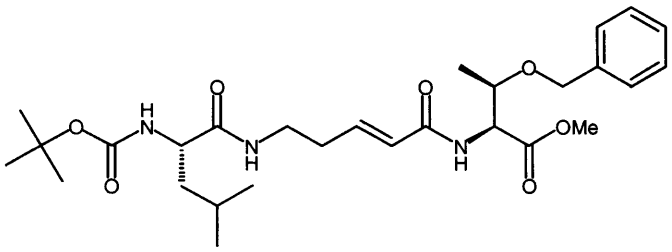
33 (39 mg, 0.11 mmol) and **19** (25 mg, 0.10 mmol) were reacted according to protocol IV yielding **39** as a brown waxy solid (43 mg, 73%).

^{1}H NMR (MeOH d_4, 400 MHz): 7.21-7.36 (m, 10 H, Ph-*H*); 6.72 (app dt, J = 15.5, J = 7.0, 1 H, CH_2CH=CH); 6.01 (d, J = 15.5, 1 H, CH_2CH=C*H*); 4.71 (dd, J = 8.5, J = 6.0, 1 H, NC*H*COOEt); 4.47-4.57 (m, 2 H, CH_2OCH_2Ph); 4.26 (m, 1 H, BocNHC*H*); 4.13 (app q, J = 7.0, 2 H, OCH_2CH_3); 3.63-3.72 (m, 2 H, $CHCH_2O$); 3.23-3.35 (m, 2 H, $NHCH_2$); 3.15 (dd, J = 6.0, J = 14.0, 1 H, CH^aH^bPh); 3.01 (dd, J = 8.5, J = 14.0, 1 H, CH^aH^bPh); 2.38 (app q, J = 7.0, 2 H, $NHCH_2CH_2$); 1.45 (s, 9 H, $C(CH_3)_3$); 1.17 (app t, J = 7.0, OCH_2CH_3). ^{13}C NMR (MeOH d_4, 100 MHz): 173.5 (C=O); 173.4 (C=O); 168.5 (C=O); 158.1 (C=O); 143.3 (CH); 139.7 (C); 138.5 (C); 130.7 (CH); 130.0 (CH); 129.8 (CH); 129.3 (CH); 129.2 (CH); 128.3 (CH); 126.3 (CH); 81.3 (C); 74.5 (CH_2); 71.5 (CH_2); 62.8 (CH_2); 56.5 (CH); 55.9 (CH); 39.7 (CH_2); 39.0 (CH_2); 31.1 (CH_2); 29.1 (CH_3); 14.8 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3423 (N-H stretch); 3059; 2980; 2936; 2870; 1722 (C=O stretch); 1678 (C=O stretch); 1499.

HRMS calc. for $C_{31}H_{41}N_3O_7$: 568.30230 $[M+H]^+$; found 568.3013.

40 – (2S,3R)-3-Benzyloxy-2-[(E)-5-((S)-2-*tert*-butoxycarbonylamino-4-methyl-pentanoylamino)-pent-2-enoylamino]-butyric acid methyl ester



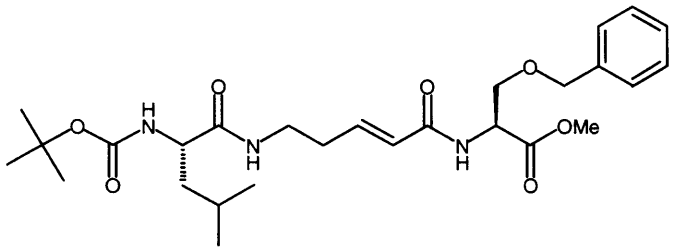
36 (32 mg, 0.11 mmol) and **35** (28 mg, 0.10 mmol) were reacted according to protocol IV. Purification yielded **40** as a pale brown waxy solid (33 mg, 61%).

^{1}H NMR ($CDCl_3$, 400 MHz): 7.16-7.29 (m, 5 H, Ph-*H*); 6.72 (app dt, *J* = 15.0, *J* = 7.0, 1 H, CH_2CH=CH); 6.45 (br s, 1 H, N*H*); 6.40 (br d, *J* = 8.5, 1 H, N*H*); 5.88 (d, *J* = 15.0, 1 H, CH_2CH=C*H*); 5.05 (br s, 1 H, N*H*); 4.69 (dd, *J* = 2.5, *J* = 9.5, 1 H, NHC*H*CO_2Me); 4.51 (d, *J* = 12.0, 1 H, C*H*^{a}H^{b}Ph); 4.32 (d, *J* = 12.0, 1 H, CH^a*H*^{b}Ph); 4.09 (dq, *J* = 2.5, *J* = 6.5, 1 H, C*H*(CH_3)OCH_2); 4.06 (br s, 1 H, N*H*CHCH_2); 3.60 (s, 3 H, OMe); 3.23-3.38 (m, 2 H, NHC*H*$_2$); 2.32 (app q, *J* = 7.0, 2 H, $NHCH_2CH_2$); 1.51-1.60 (m, 2 H, $CHCH_2CH$); 1.37-1.44 (m, 1 H, C*H*(CH_3)$_2$); 1.36 (s, 9 H, C(CH_3)$_3$); 1.16 (d, *J* = 6.3, 3 H, CH_3CHOCH_2); 0.83-0.86 (m, 6 H, CH(CH_3)$_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 173.3 (C=O); 171.6 (C=O); 166.3 (C=O); 156.3 (C=O); 141.9 (CH); 138.2 (C); 128.8 (CH); 128.2 (CH); 128.2 (CH); 125.6 (CH); 80.4 (C); 74.7 (CH); 71.2 (CH_2); 57.0 (CH_3); 53.4 (CH); 52.8 (CH); 41.7 (CH_2); 38.2 (CH_2); 32.6 (CH_2); 28.7 (CH_3); 25.2 (CH); 23.3 (CH_3); 22.4 (CH_3); 16.7 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3429 (N-H stretch); 2959; 2874; 1744 (C=O stretch); 1680 (C=O stretch); 1504.

HRMS calc. for $C_{28}H_{43}N_3O_7$: 534.3179 $[M+H]^+$; found 534.3187.

41 – (S)-3-Benzyloxy-2-[(E)-5-((S)-2-*tert*-butoxycarbonylamino-4-methyl-pentanoylamino)-pent-2-enoylamino]-propionic acid methyl ester



36 (32 mg, 0.11 mmol) and **34** (26 mg, 0.10 mmol) were reacted according to protocol IV yielding **41** as a pale brown waxy solid (39 mg, 75%).

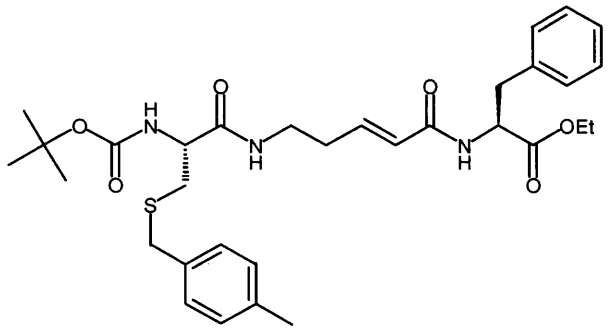
^{1}H NMR ($CDCl_3$, 400 MHz): 7.19-7.29 (m, 5 H, Ph-*H*); 6.70 (app dt, *J* = 15.5, *J* = 6.5, 1 H, CH_2CH=CH); 6.70 (br, 1 H, N*H*(Ser)); 6.59 (br s, 1 H, N*H*CH_2); 5.84 (d, *J* = 15.5, 1 H, CH_2CH=C*H*); 5.14 (br d, *J* = 6.0, 1 H, N*H*(Leu)); 4.77 (app dt, *J* = 5.0, *J* = 3.5, 1 H, NHC*H*(Ser)); 4.48 (d, *J* = 12.0, 1 H, C*H*^{a}H^{b}Ph); 4.41 (d, *J* = 12.0, 1 H, CH^a*H*^{b}Ph); 4.08 (br s, NHC*H*(Leu)); 3.84 (dd, *J* = 3.5, *J* = 9.5, 1 H, CHC*H*^{a}H^{b}O); 3.67 (s, 3 H, OMe); 3.64 (dd, *J* = 3.5, *J* = 9.5, 1 H, CHCH^a*H*^{b}O); 3.24-3.37 (m, 2 H, NHCH_2); 2.31 (app q, *J* = 6.5, 2 H, $NHCH_2CH_2$); 1.51-1.60 (m, 2 H, $CHCH_2CH$); 1.35-1.45 (m, 1 H, C*H*$(CH_3)_2$); 1.35 (s, 9 H, C$(CH_3)_3$); 0.83-0.86 (m, 6 H, CH$(CH_3)_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 173.4 (C=O); 171.4 (C=O); 170.7† (C=O); 165.7 (C=O); 164.2† (C=O); 141.8 (CH); 137.9 (C); 137.8‡ (C); 133.5† (CH); 128.9† (CH); 128.8 (CH); 128.4† (CH); 128.3 (CH); 138.1† (CH); 128.0 (CH); 125.6 (CH); 80.4 (C); 73.74† (CH_2); 73.66 (CH_2); 70.1 (CH_2); 69.8† (CH_2); 53.3 (CH); 53.04 (CH); 52.96 (CH_3); 42.5† (CH_2); 41.8 (CH_2); 38.2 (CH_2); 32.5 (CH_2); 28.7 (CH_3); 25.2 (CH); 23.3 (CH_3); 22.4 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3429 (N-H stretch); 2957; 2873; 1746 (C=O stretch); 1678 (C=O stretch); 1504.

HRMS calc. for $C_{27}H_{41}N_3O_7$: 520.3023 $[M+H]^+$; found 520.3010.

†Minor peaks resulting from diastereoisomer impurity

42 – (S)-2-{(E)-5-[(R)-2-*tert*-Butoxycarbonylamino-3-(4-methyl-benzylsulfanyl)-propionylamino]-pent-2-enoylamino}-3-phenyl-propionic acid ethyl ester



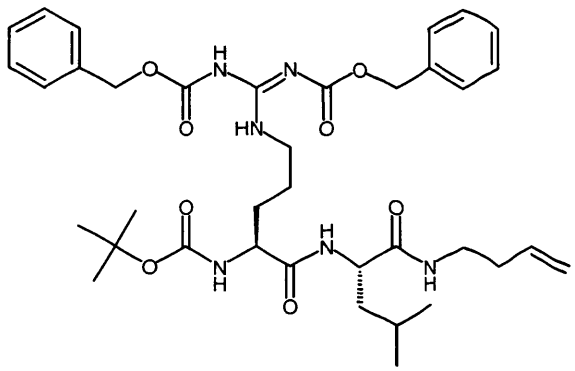
37 and **19** were reacted according to protocol IV affording **42** as a pale brown waxy solid (31 mg, 51%).

^{1}H NMR ($CDCl_3$): 7.09-7.30 (m, 9 H, Ar-*H*); 6.74 (dt, *J* = 15.0, *J* = 6.5, 1 H, CH_2CH=CH); 6.55 (br t, 1 H, NHCH_2); 6.34 (br d, *J* = 7.0, 1 H, N*H*(Phe)); 5.82 (d, *J* = 15.5, 1 H, CH_2CH=C*H*); 5.45 (br, 1 H, N*H*(Cys)); 4.91 (dt, *J* = 7.5, *J* = 6.0, 1 H, NHC*H*(Phe)); 4.27 (br m, 1 H, NHC*H*(Cys); 4.14 (app q, *J* = 7.0, 2 H, OCH_2CH_3); 3.30-3.40 (m, 2 H, $CHCH_2S$); 3.10-3.14 (m, 2 H, $CHCH_2Ph$); 2.80 (dd, *J* = 6.0, *J* = 14.0, 1 H, $CHCH^aH^bS$); 2.70 (dd, *J* = 7.0, *J* = 14.0, 1 H, $CHCH^aH^bS$); 2.36 (app q, *J* = 6.5, 2 H, $NHCH_2CH_2$); 2.35 (s, 3 H, PheMe); 1.44 (s, 9 H, $C(CH_3)_3$); 1.22 (app t, *J* = 7.0, OCH_2CH_3). ^{13}C NMR ($CDCl_3$): 171.9 (C=O); 171.0 (C=O); 165.1 (C=O); 155.6 (C=O); 141.1 (CH); 136.9 (C); 136.0 (C); 134.9 (C); 129.33 (CH); 129.29 (CH); 128.9 (CH); 128.5 (CH); 127.1 (CH); 125.2 (CH); 80.3 (C); 61.6 (CH_2); 53.8 (CH); 53.3 (CH); 38.0 (CH_2); 36.1 (CH_2); 33.9 (CH_2); 32.0 (CH_2); 30.3 (CH_2); 28.3 (CH_3); 21.1 (CH_3); 14.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3422 (N-H stretch); 2980; 2932; 2332; 1726 (C=O stretch); 1678 (C=O stretch); 1504.

HRMS calc. for $C_{32}H_{43}N_3O_6S$: 598.2951 $[M+H]^+$; found 598.2937.

43 – L-Leucinamide, N5-[bis[[(phenylmethoxy)carbonyl]amino]methylene]-N2-[(1,1-dimethylethoxy)carbonyl]-L-ornithyl-N-3-buten-1-yl



36 (500 mg, 1.76 mmol) was dissolved in DCM (10 mL) and TFA (10 mL). The mixture was monitored by LCMS until the Boc protected material could no longer be seen. Toluene (20 mL) was added and the solvents removed *in vacuo*. This was repeated once and the crude deprotected product was dissolved in DCM (4 mL) and DMF (8 mL). To a stirred solution of BocArg(diZ)OH (1.0 g, 2.0 mmol) in DMF (5 mL) was added HATU (760 mg, 2.0 mmol) and DIPEA (1 mL, 4 mmol) and the resultant solution stirred for 10 minutes The solution of crude deprotected product was added to the activated BocArg(diZ)OH solution, stirred and monitored by LCMS. The free amine was consumed after 35 min. The solvent was removed *in vacuo*, DCM (15 mL) was added and the mixture worked up as described in Protocol I yielding a cream-coloured waxy solid. The compound was triturated in ether to remove residual tetramethyl urea, yielding **43** as a cream-coloured waxy solid (413 mg, 33%).

^{1}H NMR ($CDCl_3$, 400 MHz): 9.96 (br s, (*H*N)C=N); 9.31 (br s, (*H*N)C=N); 7.30-7.45 (m, 10 H, Ph-*H*); 6.60 (d, *J* = 8.0, N*H*(Leu)); 6.28 (br s, 1 H, N*H*); 5.73 (app ddt, *J* = 17.0, *J* = 10.5, *J* = 7.0, 1 H, $H_2CC\mathit{H}{=}CH_2$); 5.58 (br s, 1 H, N*H*(Arg)); 5.27 (s, 2 H, $(C\mathit{H}_2Ph)_2$); 5.18 (AB *q*, J = 12.5, 2 H, $C\mathit{H}_2Ph$); 5.04-5.09 (m, 2 H, $\mathit{H}_2C{=}CH$); 4.32 (app dt, *J* = 9.0, *J* = 7.0, 1 H, NHC*H*(Leu)); 4.15 (app q, *J* = 6.5, 1 H, NHC*H*(Arg)); 3.90-4.05 (m, 2 H, $C\mathit{H}_2NCN_2$); 3.16-3.37 (m, 2 H, $NHC\mathit{H}_2CH_2CH{=}CH_2$); 2.22 (m, 2 H, $NHC\mathit{H}_2CH_2$); 1.60-1.70 (m, 4 H, $CHC\mathit{H}_2C\mathit{H}_2CH_2$); 1.45 (s, 9 H, $C(C\mathit{H}_3)_3$); 1.50-1.60 (m, 1 H, $C\mathit{H}(CH_3)_2$);

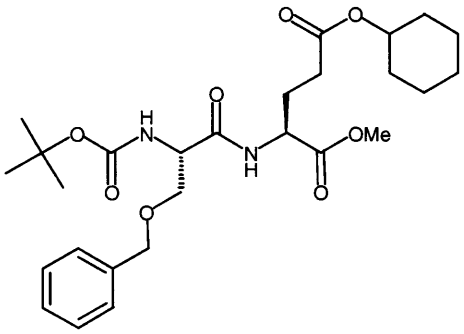
1.30-1.40 (m, 2 H, CHCH_2CH); 0.80-0.90 (m, 6 H, CH(CH_3)$_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 172.5 (C=O); 171.9 (C=O); 164.0 (C=O); 161.0 (C=O); 156.3 (C=N); 137.1 (C); 135.5 (CH); 135.0 (C); 129.32 (CH); 129.26 (CH); 128.9 (CH); 128.8 (CH); 128.4 (CH); 128.3 (CH); 117.4 (CH_2); 80.6 (C); 69.4 (CH_2); 67.5 (CH_2); 55.0 (CH); 52.3 (CH); 44.5 (CH_2); 40.8 (CH_2); 39.0 (CH_2); 34.0 (CH_2); 28.7 (CH_3); 28.4 (CH_2); 25.4 (CH_2); 25.1 (CH); 23.3 (CH_3); 22.2 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3391 (N-H stretch); 3281 (N-H stretch); 3072; 3036; 2961; 2875; 2330; 1717 (C=O stretch); 1672 (C=O stretch); 1612; 1508.

HRMS calc. for $C_{37}H_{52}N_6O_8$: 425.2440 $[M+H]^+$; found 425.2448.

$(\alpha)^{25}_D$ -22.5 (*c* 0.18 $CHCl_3$)

45 – (S)-2-((S)-3-Benzyloxy-2-tert-butoxycarbonylamino-propionylamino)-pentanedioic acid 5-cyclohexyl ester 1-methyl ester



To a stirred solution of BocGlu(OcHex)OH (2.0 g, 6.2 mmol) in methanol (10 mL) and toluene (10 mL) was added 2 M TMS diazomethane in hexanes (3.1 mL, 6.2 mmol). Acetic acid was added dropwise in until the yellow colour disappeared. The solvent was removed *in vacuo*, and a further 20 mL of toluene added and removed *in vacuo*. TFA (8 mL) and DCM (11 mL) were added and the mixture monitored by LCMS until the Boc protected material could no longer be seen. Toluene (10 mL) was added and the solvents removed *in vacuo*. This was repeated once and the crude deprotected product was dissolved in DCM (4 mL) and DMF (8 mL). To a stirred solution of BocSer(Bzl)OH (502 mg, 1.7 mmol) in DCM (4 mL) and DMF (8 mL) was added HATU (646 mg, 1.7 mmol) and DIPEA (1 mL, 4 mmol) and the resultant solution stirred for 10 min. The solution of

crude deprotected product was added to the activated BocSer(Bzl)OH solution. The resultant mixture was monitored by LCMS, and the starting material (GluO^{c}HexOMe) was consumed after 0.5 h. The solvent was removed *in vacuo*, DCM (25 mL) was added and worked up as described in Protocol I yielding **45** as a viscous colourless liquid (717 mg, 89%).

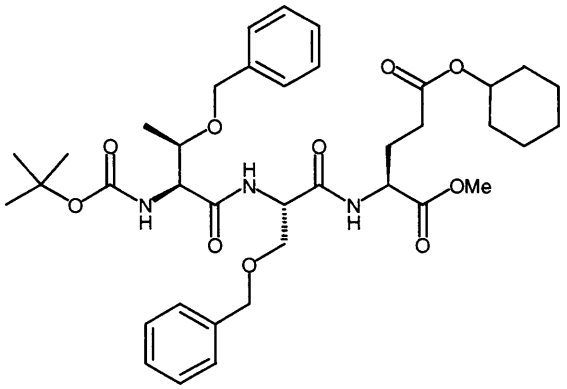
^{1}H NMR ($CDCl_3$, 400 MHz): 7.29-7.38 (m, 5 H, Ph-*H*); 7.15 (br d, *J* = 5.0, 1 H, N*H*(Glu)); 5.42 (br s, 1 H, N*H*(Ser)); 4.75 (m, 1 H, OC*H*$(CH_2)_2$); 4.67 (dt, *J* = 5.0, *J* = 8.5, 1 H, NHC*H*(Glu)); 4.55-4.61 (m, 2 H, CH_2OCH_2Ph); 3.74 (br m, 1 H, NHC*H*(Ser)); 3.93 (dd, *J* = 3.0, *J* = 9.5, 1 H, CHC*H*^{a}H^{b}O); 3.74 (s, 3 H, OMe); 3.59 (dd, *J* = 6.5, *J* = 9.5, 1 H, CHCH^a*H*^{b}O); 2.17-2.42 (m, 3 H, C*H*^{a}H^{b}CH_2CO$_2$^{c}Hex); 1.96 (app ddt, *J* = 14.0, *J* = 6.0, *J* = 8.5, 1 H, CH^a*H*^{b}CH$_2$CO$_2$^{c}Hex); 1.80-1.87 (br m, 2 H, O^{c}Hex); 1.67-1.76 (br m, 2 H, O^{c}Hex); 1.50-1.59 (br m, 1 H, O^{c}Hex); 1.47 (s, 9 H, C(CH_3)$_3$); 1.38 (br m, 2 H, O^{c}Hex); 1.21-1.32 (br m, 1 H, O^{c}Hex). ^{13}C NMR ($CDCl_3$, 100 MHz): 172.4 (C=O); 172.3 (C=O); 170.7 (C=O); 155.8 (C=O); 137.8 (C); 128.9 (CH); 128.9 (CH); 128.3 (CH); 128.25 (CH); 80.7 (C); 72.9 (CH_2); 73.4 (CH); 70.2 (CH_2); 54.4 (CH); 52.9 (CH); 52.1 (CH_3); 32.0 (CH_2); 30.9 (CH_2); 28.7 (CH_3); 28.0 (CH_2); 25.7 (CH_2); 24.1 (CH_2).

IR, solution cell, DCM (cm^{-1}): 3425 (N-H stretch); 2941; 2864; 1719 (C=O stretch); 1680 (C=O stretch); 1493.

HRMS calc. for $C_{27}H_{40}N_2O_8$: 521.2863 $[M+H]^+$; found 521.2875.

$(\alpha)^{25}_D$ +11.2 (*c* 0.59 $CHCl_3$)

46 – (S)-2-[(S)-2-((2S,3R)-3-Benzyloxy-2-(3-benzyloxy-2-tert-butoxycarbonylamino-butyrylamino)-propionylamino]-pentanedioic acid 5-cyclohexyl ester 1-methyl ester



45 (808 mg, 1.6 mmol) was dissolved in DCM (7 mL) and TFA (5 mL). The mixture was monitored by LCMS until the Boc protected material could no longer be seen. Toluene (10 mL) was added and the solvents removed *in vacuo*. This was repeated once. The resultant material was dissolved in DCM (20 mL). To astrirred solution of BocThr(Bzl)OH (525 mg, 1.7 mmol) in DMF (2 mL) was added HATU (646 mg, 1.7 mmol) and DIPEA (1 mL, 4 mmol), and the resultant solution stirred for 10 min. The solution of crude deprotected product was added to the activated BocThr(Bzl)OH solution. The resultant mixture was monitored by LCMS, and the starting material (Ser(Bzl)GluO^{c}HexOMe) was consumed after 20 min. The solvent was removed *in vacuo*, DCM (25 mL) was added and the resultant mixture worked up as described in Protocol I giving **46** as a white waxy solid (644 mg, 58%).

^{1}H NMR ($CDCl_3$, 400 MHz): 7.24-7.37 (m, 10 H, Ph-*H*); 7.20 (d, *J* = 7.2, 1 H, N*H*); 7.18 (d, *J* = 8.0, 1 H, N*H*); 5.49 (d, *J* = 6.5, 1 H, N*H*); 4.71-4.79 (m, 1 H, OC*H*$(CH_2)_2$); 4.64 (d, *J* = 11.5, 1 H, OC*H*^{a}H^{b}Ph); 4.16 (app dt, *J* = 3.0, *J* = 7.5, 1 H, NHC*H*); 4.58-4.66 (obsc, 1 H, NHC*H*); 4.55 (d, *J* = 12.0, 1 H, OC*H*^{a}H^{b}Ph); 4.51 (d, *J* = 11.5, 1 H, OCH^{a}*H*^{b}Ph); 4.45 (d, *J* = 12.0, 1 H, OCH^{a}*H*^{b}Ph); 4.30 (br m, 1 H, NHC*H*(Thr)); 4.20 (br m, 1 H, NHCHC*H*(Thr)); 3.94 (dd, 1 H, *J* = 2.5, *J* = 9.0, CHC*H*^{a}H^{b}O); 3.27 (s, 3 H, OMe); 3.50 (dd, 1 H, *J* = 6.0, *J* = 9.0, CHCH^{a}*H*^{b}O); 2.08-2.39 (m, 3 H, C*H*^{a}H^{b}C*H*$_2$CO$_2$^{c}Hex); 1.67-1.89 (br m, 5 H, CH^{a}*H*^{b}CH$_2$CO$_2$^{c}Hex, O^{c}Hex superimposed);

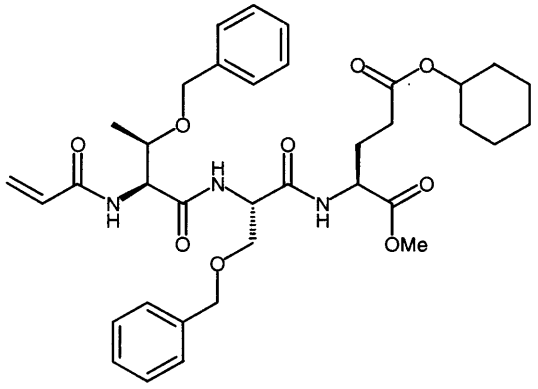
1.50-1.60 (br m, 1 H, O^{c}Hex); 1.44 (s, 9 H, C(CH_3)$_3$); 1.30-1.41 (m, 4 H, O^{c}Hex); 1.25-1.30 (obsc, 1 H, O^{c}Hex); 1.25 (d, J = 6.5, 3 H, CH_3CHOCH$_2$). ^{13}C NMR ($CDCl_3$, 100 MHz): 171.9 (C=O); 171.7 (C=O); 169.81 (C=O); 169.75 (C=O); 155.8 (C=O); 137.8 (C); 137.3 (C); 128.5 (CH);128.4 (CH); 128.0 (CH); 127.9 (CH); 127.8 (CH); 127.6 (CH); 80.3 (C); 74.8 (CH); 73.5 (CH_2); 72.9 (CH); 71.7 (CH_2); 69.3 (CH_2); 58.2 (CH); 52.9 (CH); 52.4 (CH); 51.7 (CH_3); 31.6 (CH_2); 30.5 (CH_2); 28.3 (CH_3); 27.3 (CH_2); 25.3 (CH_2); 23.7 (CH_2); 15.6 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3422 (N-H stretch); 3360 (N-H stretch); 2939; 2894; 1719 (C=O stretch); 1680 (C=O stretch); 1495.

HRMS calc. for $C_{38}H_{53}N_3O_{10}$: 712.3809 $[M+H]^+$; found 712.3814.

$(\alpha)^{25}_D$ +11.3 (*c* 0.23 $CHCl_3$)

47 – (S)-2-[(S)-2-((2S,3R)-2-Acryloylamino-3-benzyloxy-butyrylamino)-3-benzyloxy-propionylamino]-pentanedioic acid 5-cyclohexyl ester 1-methyl ester



46 (490 mg, 0.69 mmol) was dissolved in DCM (8 mL) and TFA (6 mL). The mixture was monitored by LCMS until the Boc protected material could no longer be seen. Toluene (10 mL) was added and the solvents removed *in vacuo*. This was repeated once. The resultant material was dissolved in DCM (40 mL) and triethylamine (0.12 mL, 0.76 mmol) added. Acryloyl chloride in DCM (0.17 mmol per mL) was slowly added until the starting free amine was no longer visible by LCMS. The solvent was removed *in vacuo* and the material worked up as described in protocol II, affording **47** as a white waxy solid (359 mg, 78%).

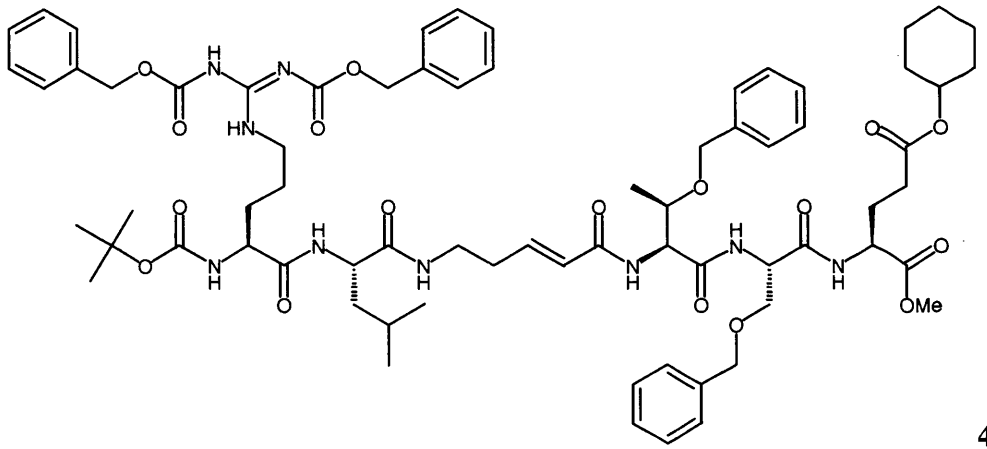
^{1}H NMR ($CDCl_3$, 400 MHz): 7.27-7.38 (m, 10 H, Ph-*H*); 7.20 (d, *J* = 7.5, 1 H, N*H*); 7.18 (d, *J* = 8.0, 1 H, N*H*); 6.64 (d, *J* = 6.5, 1 H, N*H*); 6.34 (dd, *J* = 1.0, *J* = 17.0, 1 H, H^aH^bC=CH); 6.19 (dd, *J* = 10.0, *J* = 17.0, 1 H, H_2C=C*H*); 5.71 (dd, *J* = 1.0, *J* = 10.0, 1 H, H^aH^bC=CH); 4.72-4.79 (m, 1 H, OC*H*(CH_2)$_2$); 4.73 (d, *J* = 11.5, OCH^aH^bPh); 4.69 (dd, *J* = 3.5, *J* = 6.5, 1 H, NHC*H*(Thr)); 4.59-4.66 (obsc, 1 H, NHC*H*); 4.63 (dt, *J* = 2.5, *J* = 7.5, 1 H, NHC*H*); 4.60 (d, *J* = 11.5, 1 H, OCH^aH^bPh); 4.49-4.57 (m, 2 H, OCH_2Ph); 4.23 (dq, *J* = 3.5, *J* = 6.5, 1 H, NHCHC*H*(Thr)); 3.95 (dd, *J* = 3.5, *J* = 9.0, 1 H, CHCH^aH^bO); 3.23 (s, 3 H, OMe); 3.53 (dd, *J* = 6.0, *J* = 9.0, 1 H, CHCH^aH^bO); 2.08-2.35 (m, 3 H, CH^aH^bC$H_2CO_2{}^c$Hex); 1.65-1.90 (br m, 5 H, CH^aH^bC$H_2CO_2{}^c$Hex, O^cHex superimposed); 1.50-1.65 (br m, 1 H, O^cHex); 1.30-1.45 (br m, 4 H, O^cHex); 1.23-1.30 (obsc, 1 H, O^cHex); 1.23 (d, *J* = 6.5, 3 H, CH_3CHOCH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): 172.22 (C=O); 172.17 (C=O); 170.1 (C=O); 169.6 (C=O); 166.0 (C=O); 138.1 (C); 137.8 (C); 130.6 (CH); 128.83 (CH); 128.78 (CH); 128.23 (CH); 128.21 (CH); 128.11 (CH); 128.09 (CH); 127.8 (CH_2); 75.0 (CH); 73.8 (CH_2); 73.3 (CH); 72.1 (CH_2); 69.9 (CH_2); 56.9 (CH); 53.4 (CH); 52.7 (CH); 52.1 (CH_3); 32.0 (CH_2); 30.9 (CH_2); 27.6 (CH_2); 25.7 (CH_2); 24.1 (CH_2); 15.7 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3406 (N-H stretch); 2939; 2864; 1734 (C=O stretch); 1674 (C=O stretch); 1497.

HRMS calc. for $C_{36}H_{47}N_3O_9$: 666.3390 $[M+H]^+$; found 666.3370.

$(\alpha)^{25}{}_D$ +11.9 (*c* 0.25 $CHCl_3$)

L-Glutamic acid, N5-[bis[[(phenylmethoxy)carbonyl]amino]methylene]-N2-[(1,1-dimethylethoxy)carbonyl]-L-ornithyl-L-leucyl-(2E)-5-amino-2-pentenoyl-O-(phenylmethyl)-L-threonyl-O-(phenylmethyl)-L-seryl-, 65-cyclohexyl 61-methyl ester



48

43 (78 mg, 0.11 mmol) and **47** (67 mg, 0.10 mmol) were reacted according to protocol IV in the Biotage Initiator 60 microwave unit yielding **48** as a pale brown waxy solid (51 mg, 38%). The HPLC yield had been determined to be 67% by ELSD.

^{1}H NMR ($CDCl_3$, 500 MHz): 9.46 (br s, 1 H, *H*NC=N); 9.29 (br s, 1 H, *H*NC=N); 7.69 (d, *J* = 8.0, 1 H, N*H*(Ser)); 7.55 (br d, 1 H, N*H*(Thr)); 7.38 (obsc, 1 H, N*H*(Glu)); 7.16-7.41 (m, 20 H, Ar-*H*); 6.76 (br t, 1 H, N*H*CH_2); 6.70 (ddd, *J* = 6.0, *J* = 9.1, *J* = 15.4, 1 H, CH_2CH=CH); 6.69 (d, *J* = 8.9, 1 H, N*H*(Leu)); 5.98 (d, *J* = 15.4, 1 H, CH_2CH=C*H*); 5.94 (br, 1 H, N*H*(Arg)); 5.15-5.25 (m, 4 H, $2CO_2CH_2$); 4.80 (m, 1 H, NHC*H*(Ser)); 4.72 (m, 1 H, OC*H*$(CH_2)_2$); 4.47, 4.51 (AB q, *J* = 11.8, 4 H, $2OCH_2$Ph); 4.52 (dd, *J* = 2.1, *J* = 6.5, 1 H, NHC*H*(Thr)); 4.57 (dt, *J* = 8.3, *J* = 5.4, 1 H, NHC*H*(Glu)); 4.20-4.30 (m, 2 H, NHC*H*(Leu), NHCHC*H*(Thr) superimposed); 4.05 (app q, *J* = 6.5, 1 H, NHC*H*(Arg)); 3.84-3.90 (m, 1 H, $CH^aH^bNCN_2$); 3.86 (dd, *J* = 5.4, *J* = 9.5, 1 H, $CHCH^aH^bO$); 3.72-3.79 (m, 1 H, $CH^aH^bNCN_2$); 3.69 (s, 3 H, OMe); 3.63 (dd, *J* = 5.2, *J* = 9.5, 1 H, $CHCH^aH^bO$); 3.45-3.52 (m, 1 H, $CONHCH^aH^b$); 2.97-3.07 (m, 1 H, $CONHCH^aH^b$); 2.40-2.50 (m, 1 H, $CH_2CH^aH^bCO_2{}^c$Hex); 2.21-2.37 (m, 3 H, $NHCH_2CH_2$, $CH_2CH^aH^bCO_2{}^c$Hex

superimposed); 2.09-2.20 (m, 1 H, C*H*^{a}H^{b}CH$_2$CO$_2$^{c}Hex); 1.80-1.91 (m, 1 H, CH^{a}*H*^{b}CH$_2$CO$_2$^{c}Hex); 1.77-1.85 (br m, 2 H, O^{c}Hex); 1.68-1.72 (br m, 2 H, O^{c}Hex); 1.45-1.55 (br m, 6 H, CHC*H*$_2$C*H*$_2$CH$_2$ (4 H), O^{c}Hex (1 H), NHCHC*H*(CH$_3$)$_2$ superimposed); 1.42 (s, 9 H, C(C*H*$_3$)$_3$); 1.2-1.4 (br m, 5 H, O^{c}Hex); 1.22 (d, *J* = 6.4, 3 H, C*H*$_3$CHOCH$_2$); 0.82 (d, *J* = 6.2, 3 H, CHC^{a}*H*$_3$C^{b}H$_3$); 0.76 (d, *J* = 6.2, 3 H, CHC^{a}H$_3$C^{b}*H*$_3$). ^{13}C NMR ($CDCl_3$, 125 MHz): 172.7 (C=O); 172.3 (C=O); 171.84 (C=O); 171.77 (C=O); 170.3 (C=O); 170.0 (C=O); 166.9 (C=O); 163.6 (C=O); 160.9 (C=O); 156.1 (C=O); 155.7 (C=O); 140.6 (CH); 138.3 (C); 137.7 (C); 136.8 (C); 134.5 (C); 128.9 (CH); 128.8 (CH); 128.5 (CH); 128.4 (CH); 128.3 (CH); 128.2 (CH); 127.92 (CH); 127.87 (CH); 127.7 (CH); 127.6 (CH); 127.5 (CH); 127.3 (CH); 126.4 (CH); 80.3 (C); 74.5 (CH); 73.0 (CH); 72.8 (CH_2); 71.7 (CH_2); 69.2 (CH_2); 69.0 (CH_2); 67.0 (CH_2); 58.4 (CH); 55.5 (CH); 52.8 (CH); 52.3 (CH_3); 51.7 (CH); 51.4 (CH); 44.3 (CH_2); 40.3 (CH_2); 37.9 (CH_2); 31.5 (CH_2); 30.83 (CH_2); 30.76 (CH_2); 28.3 (CH_3); 27.34 (CH_2); 27.25 (CH_2); 25.3 (CH_2); 25.1 (CH_2); 24.6 (CH); 23.7 (CH_2); 22.8 (CH_3); 21.5 (CH_3); 16.1 (CH_3).

IR, solution cell, DCM (cm^{-1}): 3387 (N-H stretch); 3055; 2936; 2862; 2307; 1719 (C=O stretch); 1670 (C=O stretch); 1612; 1504.

HRMS calc. for $C_{71}H_{95}N_9O_{17}$: 1346.6924 $[M+H]^+$; found 1346.6952.

4.2 – Peptide Synthesis

4.2.1 – Materials and Methods

All amino acids were Fmoc protected. The side-chain protection of Fmoc amino acids is shown in Table 1.

Amino Acid	Side-Chain Protection
Serine	^{t}Bu
Threonine	^{t}Bu
Cysteine	Trityl
Glutamic Acid	^{t}Bu
Aspartic Acid	^{t}Bu
Asparagine	Trityl
Tyrosine	^{t}Bu
Arginine	Pbf

Table 1 – Side-chain protective groups
(Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)

Amino acids, HBTU and HOBt were purchased from CS Bio Co., ultra-pure DMF from AGTC Bioproducts Ltd., NMO from Rathburn Chemicals Ltd. and piperidine and DIPEA from Iris Biotech. All other reagents were obtained from Aldrich and were used as received.

Chlorotrityl resins were obtained from CS Bio Co. or GL Biochem Ltd., *N*-methyl amino polystyrene resins polystyrene resins from Novabiochem.

Peptide fragments were synthesized using a CEM Discover microwave peptide synthesizer and a CEM Liberty solvent-handling unit. Preparative HPLC and HPLC analyses and were carried out on a Varian ProStar system. Analytical HPLC was performed on Varian Microsorb MV 100-5 C18 150x4.6 mm column, eluting with H_2O

(containing 0.1% TFA, 'A') and acetonitrile (containing 0.1% TFA, 'B'). The column was flushed with 95:5 A:B, isocratic flow continuing for 2 min. The percentage B was increased to 95% over 6 min, followed by 6 min isocratic flow. Preparative HPLC was performed on Phenomenex Jupiter 10μm C18 250x10.0 mm column, eluting with A and B. Two methods were used. The first was the same as for the analytical HPLC. In the second, the column was flushed with 75:25 A:B. The percentage B was increased to 95% over 12 min, followed by 2 min isocratic flow. Mass spectra were obtained using a Micromass Quattro.

4.2.2 – Typical resin handling procedures

Resins were always stored shrunk at RT. Shrinkage was achieved by addition of ether, shaking, draining and removal of solvent under vacuum for 3 h.

4.2.2.1 – Resin Wash

A resin wash comprised (unless otherwise stated) three washes with DMF, followed by three with DCM. In each wash enough solvent was used to sufficiently submerge the resins, and the resins were shaken (ca. 10 s) prior to drainage and addition of the next round of washing solvent.

4.2.2.2 – Fmoc Test

To check the loading of resins, a small portion of the loaded, shrunk resins (3-5 mg) were removed and piperidine in DMF (1 ml, 1:4 v:v) added. After 1 h, 0.5 ml of the solution was removed, made up to 5 ml with piperidine in DMF (1 ml, 1:4 v:v), and the solution analysed by UV to calculate the loading in mmol/g.

4.2.2.3 – Kaiser Test

The Kaiser[127] test for free amines was carried out as follows. Solutions of ninhydrin in ethanol (5% w/v, 2 drops), phenol in ethanol (4:1 w/v, 2 drops) and KCN in pyridine (2% of 1mmol/l aqueous solution v:v, 2 drops) were added to the resins and the resultant mixture heated to 80 °C for 5 minutes. A blue colour is indicative of the free amine.

4.2.2.4 – Fmoc Deprotection

The resins were swollen in DMF for 1 h. Sufficient piperidine:DMF (1:5 v:v) to completely immerse the resins was added and the mixture left to stand for 2 mins. The deprotection solution was drained, and the resins washed with DMF (3x2 ml). Piperidine:DMF (1:5 v:v) was added to the resins and the mixture left to stand for 8 mins with occasional agitation. The solution was drained and the resins washed.

4.2.3 – Preparation of Resins

4.2.3.1 – Typical loading of chlorotrityl resins

Chlorotrityl resins (1 mmol/g, 0.5 g) were swollen in DMF (10 ml) for one hour, and then washed. DCM (10ml), acetic acid (0.25 mmol, 0.015 ml) and DIPEA (0.25 mmol, 0.043 ml) were added, and the resins left to stand for 2 h, with occasional agitation. After a resin wash, Fmoc amino acid (1 mmol) and DIPEA (1 mmol, 0.17 ml) were added to DMF (10 ml) and the mixture stirred until complete dissolution of the amino acid had occurred. This solution was then added to the resins and the resultant mixture left to stand for 4 h, with occasional stirring. The resins were washed, shrunk and their loading determined by the Fmoc test. The resins were then swollen in DMF (10 ml) for one hour and washed with DCM:MeOH:DIPEA (17:2:1 v:v:v, 3x10 ml). Finally the resins were washed and shrunk for storage.

4.2.3.2 – Preparation of sulfonamide safety-catch linker resins

Aminomethyl polystyrene resins (1.13 mmol/g, 5.0 g) were swollen in DMF (15 ml) for one hour, and then washed with DIPEA in DMF (3 drops/5 ml, 3x15 ml) and DCM (3x15 ml). Acetic anhydride (0.32 g, 3.15 mmol) in DCM (15 ml), DIPEA (1.1 ml, 6.0 mmol) and DMAP (38 mg, 0.25 mmol) were added to the resin, and the resultant mixture left to stand for 1 h with occasional stirring, after which time the resins were washed. 4-Sulfamylbutyric acid (1.25 g, 7.5 mmol) and HOBt (1.0 g, 7.5 mmol) were dissolved in DMF (15 ml), added to the resins and the resultant mixture shaken for 1 h. DIC (1.16 ml, 7.5 mmol) was then added and the resins shaken for 5 h and washed.

4.2.3.3 – Loading of safety-catch linker resins

FmocLeu fluoride was prepared from FmocLeu (2.8 g, 8.0 mmol) as described in the literature.[32] The resultant compound was dissolved in DCM (12 ml). The resultant solution (6 ml) and DIPEA (0.87 ml, 5.0 mmol) was added to the sulfonamide safety-catch linker resins and the mixture left to stand for 18 h with occasional agitation. The resins were washed, and checked for loading. Loading was 0.25 mmol/g, and so the loading procedure was repeated, increasing the loading to 0.40 mmol/g.

4.2.4 – Microwave-enhanced SPPS

Peptide fragments were synthesized on a 0.1 mmol scale. 0.2 M amino acid solutions were used. Coupling solution consisted of 0.5 M HBTU/HOBt in DMF, activator base solution 2 M DIPEA in NMP and deprotection 20% piperidine in 0.1 M HOBt/DMF. Default cycles were used unless a double or triple coupling was required, in which case appropriate extra steps were added.

Prior to automated SPPS resins were swollen in DMF for 1 h. the loadings of resins were as follows – FmocLeu-Chlorotrityl, 0.50 mmol/g; FmocGly-Chlorotrityl, 0.16 mmol/g; FmocAsn-Chlorotrityl, 0.16 mmol/g; FmocLeu-Safety-catch, 0.40 mmol/g.

4.2.5 – Cleavage Protocols

4.2.5.1 – TFA Cleavage

TFA cleavage was carried out on small samples of resins, typically 20 mg. The resins were swollen in DMF for 1 h, deprotected and washed. Sufficient cleavage solution (TFA:H_2O:EDT:TIS, 94:2.5:2.5:1 v:v:v:v) to immerse the resins was added and the resultant mixture was left for 4 h with occasional agitation. The crude cleavage mixture was drained, and the resins washed once with TFA and three times with DCM. All solutions were combined and concentrated to ~0.5 ml under reduced pressure. In cases where a precipitate had formed, 2/3 drops of DCM were added and this gave a homogenous solution. The resultant solution was added to chilled ether (1 ml), and the mixture centrifuged. The supernatant was discarded and ether (1 ml) added to the pellet. Dispersion of the pellet was achieved by sonication (10 min), and the mixture centrifuged

again. This process was repeated twice to remove unwanted deprotection byproducts. The product was then analysed using HPLC and mass spectrometry.

4.2.5.2 – Cleavage from Safety Catch Linkers

Cleavage from safety-catch linkers with homoallyl amine was achieved using the literature precedent,[32] substituting homoallyl amine for the thiol. The solvent was removed *in vacuo* yielding the protected peptide. To prepare for HPLC analysis, 1.5 ml cleavage solution (TFA:H_2O:EDT:TIS, 94:2.5:2.5:1 v:v:v:v) added and the mixture left to stand for 4 h. The resultant solution was concentrated and treated in the same manner as the TFA-cleaved peptides.

4.2.5.2 – Cleavage with Acetic Acid

Acetic acid cleavage was carried out on ~100 mg of resins, typically. The resins were swollen in DMF for 1 h, deprotected and washed. To the deprotected resins was added Boc anhydride (10 equivalents in DCM). The resultant mixture was left to stand for 2 h with occasional agitation. The solution was the drained and the resins washed. The cleavage mixture (AcOH:TFE:DCM, 1:1:8 v:v:v) was added and the mixture left to stand for 2 h with occasional agitation. The resultant solution was separated and the resins washed with DCM. The combined solutions were washed twice with saturated $NaHCO_3$, dried with $MgSO_4$ and the solvent removed *in vacuo* yielding the cleaved, fully protected peptide.

4.2.6 – Manual SPPS

Where a manual coupling was required, a solution of amino acid (5.5 equiv.), HOBt (5 equiv.), HBTU (5 equiv.) in a minimum volume of DMF was added to the swollen resins. If necessary, extra DMF was added to submerge the resins. DIPEA (5 equiv.) was then added and the mixture occasionally agitated. Every 2 h a few resin beads were removed, washed, and checked using the Kaiser test. When coupling was complete, the resins were washed.

4.2.7 – On-Resin N-Acryloylation

The resins were swollen in DMF for 1 h, deprotected and washed. To the deprotected resins were added acryloyl chloride (2 equivalents in DCM at 0.67 M) and DIPEA (2 equivalents). The resultant mixture was left to stand, with occasional agitation, and the Kaiser test was employed every 2 h. When acryloylation was complete the resins were washed.

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